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Learning and Memory in Invertebrates: Mollusks

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Introduction

Over the past several decades, it has become evident that fundamental mechanisms of learning and memory are remarkably conserved among animals, ranging from humans and other higher mammals to lower vertebrates and invertebrates, such as fruit flies and mollusks. Molluscan species in particular have proven quite useful for cellular and molecular studies; they exhibit several types of learning, including non-associative forms (sensitization, habituation, etc.) and associative forms (classical conditioning and operant conditioning). Because of the relative simplicity of their nervous systems, mollusks provide powerful models for examining the mechanisms of the induction and maintenance of memory. One of the main strengths of the molluscan systems is the ability to make direct links between the animal's behavioral plasticity and its underlying cellular and molecular mechanisms. Because of the space limitations of this review, we are unable to discuss the contributions of the numerous molluscan model systems (such as the terrestrial snail, *Helix*; the marine mollusk, *Tritonia*; and the pond snail, *Limax*), each of which exhibits similar forms of learning to those we will describe; nor will we discuss the cephalopods (octopus, squid, and cuttlefish), which have complex nervous systems and are capable of more advanced forms of training, including spatial learning and observational learning. Rather, we will highlight three molluscan species that have made significant contributions to our understanding of the cellular and molecular mechanisms of learning and memory: the marine mollusk, *Aplysia californica*; the nudibranch, *Hermisenda crassicornis*; and the pond snail, *Lymnaea stagnalis*.

Aplysia

Of the mollusks, *Aplysia* is perhaps the most extensively studied in the field of learning and memory. Because of the relative simplicity of its nervous system and the animal's large size, *Aplysia* is particularly amenable to combined behavioral, circuit, and cellular analyses. *Aplysia* exhibits several forms of memory, including both associative and nonassociative forms. Two behavioral systems have been the main

focus of research in *Aplysia*: (1) the defensive withdrawal reflexes of the tail, siphon, and gill and (2) the feeding system. Multiple forms of memory have been studied in each. The withdrawal reflexes, which have been the most extensively studied, exhibit nonassociative forms of memory, including sensitization and habituation, as well as classical conditioning. The feeding system can also be conditioned using both operant and classical procedures. Very recently, it has also been shown that the gill reflex can also be operantly conditioned. The fortuitous finding that multiple forms of memory can be induced by a single simple circuit offers a unique opportunity to compare the cellular and molecular mechanisms that mediate different forms of memory in the same system.

Sensitization

The defensive withdrawal reflexes of the *Aplysia* tail, siphon, and gill show both sensitization, an increase in behavioral responsiveness following a noxious stimulus, and habituation, a decrement in a behavioral responsiveness following repeated, weak stimulation. In response to a weak tactile stimulation to the body wall or tail, *Aplysia* reflexively withdraws its gill, siphon, and tail. A strong, noxious stimulation, such as shock (e.g., to the tail or head), sensitizes these reflexes, producing an increase in their magnitude and duration in response to subsequent weak, tactile stimuli. Conversely, weak low-frequency stimulation habituates these reflexes. Although important research on habituation is currently being conducted, we will restrict the discussion here to sensitization because its mechanisms are better understood.

The experimental preparation often used is the tail-elicited siphon withdrawal (T-SWR). The amount and pattern of training trials is important in determining the type and the duration of the memory induced in many systems, ranging from insects to mammals. The efficacy of several training patterns has been explored in *Aplysia*. For example, a single tail shock induces short-term memory (STM) for sensitization, which lasts <30 min. Multiple (four or five) trials spaced by 15 min induces two long-lasting forms of memory: intermediate-term memory (ITM) that lasts 90 min–3 h, and long-term memory (LTM) that begins to be expressed about 10 h after training and lasts at least 2 days. Massed training, in which the same total amount of training is given but without any intervening intertrial intervals (ITIs), does not induce LTM, and induces only an early decaying form of ITM. Finally, multiple trials spaced over several days (one trial per day for 4 days) induce LTM that lasts several weeks.

Another training parameter that affects memory induction is neuronal activity. In the sensitization experiments described above, one site on the tail is used to test the reflex, and shock is applied to another site; thus the sensory neurons (SNs) at the test site do not fire during the tail shock. This form of sensitization is termed 'repeated-trial' (RT) sensitization. However, the shock site itself also shows sensitization, and this 'site-specific' sensitization differs from RT sensitization in that ITM induction requires only a single shock.

Related to the relationship between trial number and pattern is the question of how a prior learning experience influences later training. Recently, two forms of latent learning have been described in the T-SWR. Latent learning is a phenomenon in which learning-related changes occur, but these changes are subthreshold for detection by memory tests. The presence of latent learning is typically revealed as a facilitation of subsequent memory formation, often called savings. In one study, LTM was induced and then allowed to decay. After the T-SWR returned to baseline (the memory was 'forgotten'), animals were given stimulation that typically produces no learning. In previously trained animals, this stimulus pattern induced both ITM and LTM. In the second study, LTM was induced ipsilaterally, after which, animals were given a brief shock that is below threshold for induction of sensitization in otherwise untrained animals. This stimulus, given to the contralateral side, induced sensitization in previously trained animals.

Cellular and molecular mechanisms A major focus of recent research in *Aplysia* has been to relate the pattern of sensitization training to the molecular mechanisms involved in the induction and expression of memory. These experiments have been carried out using *in vitro* and semi-intact preparations. RT memory and site-specific memory have different requirements for protein kinase A (PKA) and protein kinase C (PKC) as well as different requirements for translation and transcription. RT-ITM is dependent on PKA and requires protein synthesis, and RT-LTM requires both translation and transcription, plus mitogen-activated protein kinase (MAPK) activation and tyrosine kinase activity. Site-specific ITM differs from RT-ITM in that it is dependent on PKC (PKM) and does not require protein synthesis. However, like RT-ITM, it requires MAPK activation.

A more thorough investigation of the molecular mechanisms of ITM and LTM has been achieved using a cellular model of sensitization, facilitation of tail SN-motor neuron (MN) synapses. Exogenous application of serotonin (5-HT), a neurotransmitter released by tail shock or tail nerve shock induces

facilitation of SN-MN synapses. A single pulse of 5-HT induces short-term facilitation (STF) that lasts <30 min and requires activation of PKA (or PKC if the synapse has been previously depressed). Five pulses of 5-HT induce both intermediate-term facilitation (ITF), that lasts about 90 min and requires synthesis of new protein, and long-term facilitation (LTF) that lasts >24 h and requires both translation and gene transcription. Morphological changes in SN structure, including an increase in the number of varicosities, accompany LTF induction and are required for the maintenance of LTF from 24 to 72 h. The mechanisms of LTF have been widely studied and are described in depth elsewhere. Briefly, activation of PKA activates MAPK, ultimately resulting in cyclic AMP-responsive element binding protein (CREB)-mediated transcription that synthesizes immediate early genes (IEGs). Examples of IEGs that are important for the induction of LTF include CCAAT enhancer-binding protein (C/EBP) and ubiquitin hydrolase (Ap-Uch). Epigenetic mechanisms such as direct modification of DNA or histones have recently been found to be important for long-lasting synaptic modifications, both in vertebrate and in invertebrate models of learning and memory. In *Aplysia*, increasing histone acetylation facilitates the induction of LTF. Finally, secreted factors also appear to have a role in the induction of LTF. Both transforming growth factor-beta (TGF- β) and a TrkB ligand are involved, as well as an SN-specific peptide, sensorin. Not much is known about ITF, but this midrange form of memory seems to utilize some of the same players as LTF. For example, induction of ITF also requires both PKA and MAPK activation.

Synaptic correlates have also been found for site-specific memory. In site-specific memory, the test site is activated during training; thus, the cellular correlate for site-specific memory involves pairing a pulse of 5-HT with direct SN activation with current injection. This correlate is called activity-dependent (AD) facilitation, and a single pairing induces both ITF and LTF. As with the two different forms of memory, the two corresponding forms of facilitation have different molecular requirements. AD-ITF requires PKC, not PKA, for induction, and it does not require protein synthesis.

Associative Forms of Learning

Associative forms of memory include both classical, or Pavlovian, conditioning and operant conditioning. Classical conditioning is a form of learning in which a nominally neutral stimulus (conditioning stimulus, CS), when paired with a reinforcing stimulus (unconditioned stimulus, US) that causes an unconditioned reflexive response, gains the ability to cause the

response itself. When the CS and US are paired, the animal learns to associate the CS with the US such that, after training, the animal then responds to the CS alone. Operant conditioning is similar except that the animal learns to associate its own behavior with a reinforcer. Both classical and operant conditioning can be induced in the feeding system in *Aplysia*. The fact that both of these forms of learning impact the same circuit presents an opportunity to compare the cellular and molecular mechanisms underlying them.

Classical Conditioning

Classical conditioning in *Aplysia* was first described for the T-SWR, and its mechanisms were found to involve AD synaptic facilitation. More recently, classical conditioning has been investigated in the feeding system, in which tactile stimulation of the lips (CS) is paired with ingestion of food (US). Pairing of the tactile stimulation with seaweed leads to an increase in the number of bites the *Aplysia* makes in response to presentation of the CS alone. Ten trials spaced by 4 min induces memory in the form of an increase in the number of bites measured 1 h and 24 h after training.

Cellular mechanisms The cellular mechanisms of conditioning can be investigated in *in vitro* preparations in which the CS and US are substituted by nerve stimulation: stimulation of the antennal nerve, AT4 substitutes for the CS, and stimulation of a branch of the esophageal nerve, En2, substitutes for the US. The responses of central pattern generator (CPG) neurons to the CS are altered after paired training: B4/5 (increased number of ingestion-like buccal motor patterns (BMPs)), B31/32 (increase in the peak amplitude and net size of complex excitatory postsynaptic potentials (EPSPs)), and CB1–2 (increase in the number of action potentials).

Operant Conditioning

Operant conditioning in *Aplysia* was first described in the head-waving system. Three other forms of operant conditioning have since been described in *Aplysia*: aversive and appetitive feeding responses, and more recently, it has been found that the gill-withdrawal reflex can also be operantly conditioned. For appetitive operant conditioning, seaweed is presented to *Aplysia*, either with forceps or in a plastic net with a hole in it. The animal bites the food, pulls it into its buccal cavity, and swallows it. Since swallowing is the reinforcing stimulus, food is provided to the animal as long as it continues to feed.

In aversive conditioning, no holes are made in the net, so the food cannot be swallowed. After several

failed swallowing attempts, the food is rejected. Over the training period, the animal decreases the frequency of its swallowing attempts. A single 5 min training session induces very short-term memory that lasts only 15 min and LTM that is expressed 12–24 h after training, a longer training session that lasts until cessation of responsiveness induces STM that lasts 30–60 min, and, finally, multiple training sessions (3×5 min, 30 min ITI) induce ITM that lasts up to 4 h. These results demonstrate that different phases of memory can be induced independently as one 5 min training session induces very short-term memory and LTF in the absence of STF and ITF. A requirement for protein synthesis for the induction of LTF is suggested by the finding that of the four described memory phases, only LTM was blocked by cooling the animal for 15 min immediately after training.

Aplysia has also been a useful model system in which to examine the modulatory effects of circadian rhythms on induction of memory. *Aplysia californica*, a diurnal species, shows greater memory for operant conditioning (as well as sensitization) when trained and tested during the day as opposed to at night; conversely, the nocturnal species, *Aplysia fasciata* exhibited improved memory when trained and tested at night.

Cellular mechanisms *In vitro* operant conditioning is induced by En2 stimulation (reinforcer) given, contingent upon spontaneous production of either ingestion-like behavior (appetitive conditioning) or egestion-like behavior (aversive). Thus, activity in a specific nerve, En2, acts as a general reinforcer during conditioning of feeding responses.

One CPG neuron, B51, has been found to be a site of modulation during operant learning. Activity in B51, a member of the CPG that produces BMPs, correlates with the occurrence of the ingestion-like motor patterns that can be induced by stimulation of nerve n2,3. B51's that have received contingent reinforcement show greater input resistance, an increase in the occurrence of plateau potentials, and decreased burst thresholds, but showed no change in V_m relative to either yoked or untrained controls.

Convergence of Different Forms of Memory Processing

Now that the mechanisms of various associative and nonassociative forms of memory have been described at the behavioral and cellular levels, it is possible to compare them on a mechanistic level. The first analysis of this sort involved comparing the cellular effects classical conditioning on interneuron B51 to what was already known for operant conditioning (see above). As in operant conditioning, classical

conditioning led to an increase in the number of CS-evoked plateau potentials; however, in contrast to operant conditioning, classical conditioning caused no significant change in input resistance, and in fact there was an increase in plateau potential threshold. Thus, although both types of conditioning result in an increase in the number of plateau potentials in B51, the mechanisms are not the same.

Another site of convergence of operant and classical conditioning is the requirement for dopamine (DA). DA plays an essential role in reward learning, both in vertebrates and in invertebrates. DA was found to be required for *in vitro* models of both operant and classical conditioning.

Over the last several decades, a wealth of information has been learned about several types of memory formation in *Aplysia*. The mechanisms of both associative and nonassociative forms of memory have been studied at the behavioral, synaptic, and molecular levels. As researchers continue to learn more about these mechanisms, they can also now begin to compare the signaling pathways involved between different types of associative memory, for example, between operant and classical conditioning in the feeding behavior (see above), and also between associative and nonassociative forms; for example, it has recently been shown that the gill-withdrawal reflex, long known to exhibit sensitization and habituation, also demonstrates operant conditioning.

Hermisenda

Rotation-induced inhibition of phototaxis in the nudibranch mollusk *Hermisenda* has been a powerful model for the study of classical conditioning. The circuitry involved in the behavior is more complex than either of the *Aplysia* circuits; however, the easily accessible sensory neurons (type B photoreceptors) are a site of convergence for the CS (light) and US (rotation) pathways, providing an excellent opportunity to examine the cellular and molecular mechanisms underlying the interaction of these two signals. Prior to conditioning, *Hermisenda* display a phototactic response by locomoting to a light source (CS). Conversely, when shaken or rotated vigorously (US), the animal contracts its foot and ceases locomotion. After CS-US pairing, *Hermisenda* exhibit two conditioned responses to the CS alone: (1) inhibition of light-induced locomotion and (2) foot shortening. This behavior has been extensively studied both experimentally and with computational models, and it has been found to share many of the characteristics of classical conditioning seen in mammals, including extinction and savings.

Recent studies have focused on two mechanistic levels. First, ongoing examination of the primary

sensory neurons continues to further elucidate the cellular and molecular mechanisms of the convergence of the CS and US signals at the type B photoreceptors. Second, progress is being made to identify interneurons and motor neurons downstream of the primary SNs, and to determine (1) how their circuit and cellular properties are altered by conditioning, and (2) how these changes contribute to changes in conditioned behavior.

Memory for light-induced inhibition of phototaxis can exist in several temporal domains. One or two pairings induce STM, which lasts <10 min and requires activation of PKC. Multiple pairings induce longer-lasting forms of memory: ITM, which lasts about 2.5 h and requires protein synthesis, and LTM, which lasts ≥ 1 day and requires both translation and transcription. Recently, two forms of LTM have been differentiated mechanistically; consolidated LTM (CLTM) (which lasts up to 3 days) can be distinguished from LTM (which lasts only about 24 h) by its requirement for a second round of protein and RNA synthesis, and by its disruption by the arginyl-glycyl-aspartate (RGD) peptide, a competitive inhibitor of integrins.

One characteristic of LTM that is currently a subject of much investigation in mammalian systems, as well as in invertebrates, is its susceptibility to disruption by protein synthesis blockers during reactivation by the CS alone, a process called reconsolidation. Evidence for reconsolidation has been demonstrated in LTM in *Hermisenda*. Animals were given LTM training, followed a few hours later by a single retrieval test (CS alone) to reactivate the memory. If this test was followed by treatment with a protein synthesis blocker (anisomycin) within 10–30 min, LTM was not induced. Similar results were obtained if transcription was blocked with actinomycin or if bond formation between cell adhesion molecules (CAMs) was blocked with the RGD peptide.

Cellular and Molecular Mechanisms

Light activates rhodopsin in the cell membranes of two types of photoreceptors of the *Hermisenda* eye: type A and type B. The US, shaking or rotation of the animal, excites hair cells (HCs) in the statocysts. As mentioned earlier, the first site of convergence of the CS and US is at the primary SNs, namely the B-type photoreceptors. The HCs synapse onto the type B photoreceptors both directly through GABAergic synapses and indirectly via as yet unidentified serotonergic interneurons. After CS-US pairing, photoreceptors exhibit increased excitability, increased input resistance, increased firing frequency, and increased generator potentials in response to light. These cellular

changes are also observed in *in vitro* preparations using a single trial conditioning procedure in which light (CS) is paired with 5-HT application (US). We will focus here on the molecular mechanisms of convergence at the type B photoreceptors, though it should be kept in mind that there are further sites of CS-US interaction, including the first level of interneurons.

In the type B photoreceptors, the main effect of the combined CS and US signals is considered to be an enhanced increase in internal Ca^{2+} via activation of two different signaling pathways, which induces a persistent activation of PKC. Light activates rhodopsin on the B photoreceptor membrane, which, via G-protein interactions, activates a signaling cascade through PLC, DAG, IP3, and the IP3 receptor to release Ca^{2+} into the cytoplasm from endoplasmic reticulum (ER) stores. Activation of the HCs by shaking or rotation causes release of GABA from the HCs, which binds metabotropic GABA receptors on the photoreceptor and leads to Ca^{2+} release from intracellular stores through mechanisms that are not entirely known but are thought to involve phospholipase C (PLC) activation. In addition, HCs also make excitatory serotonergic connections onto the type B photoreceptors, probably through a polysynaptic pathway. 5-HT activates MAPK, through both PKC-dependent and PKC-independent pathways.

Accompanying the long-term physiological changes is a change in the morphology of the type B photoreceptors. Multiple paired training trials induce contraction of the terminal branches of the type B photoreceptors. This morphological response is sensitive to ryanodine receptor (Ryr) blockers and therefore is dependent on Ca^{2+} release from internal stores. The functional significance of the reduction in arborization is not yet clear.

Downstream Effectors of Transcription and Translation

Much of the focus of the molecular mechanisms of ITM and LTM in *Hermisenda* has been on the events occurring upstream of transcription. However, the protein products of conditioning-induced translation and transcription are currently being investigated. One class of molecules that appear to be necessary for intermediate- and long-term changes in synaptic efficacy is involved in actin stability. Cytoskeleton-related proteins play important roles in cell morphology and in the composition of the cell membrane, and the activity of several of these have been found to be altered by conditioning in *Hermisenda*. Conditioned stimulus pathway phosphoprotein 24 (Csp24) is a cytoskeletal-related protein with several actin-binding domains that are upregulated and phosphorylated by procedures that induce intermediate-term and long-term increases in

excitability, and it is associated with a decrease in a specific potassium current, I_A . In single trial conditioning, 5-HT mediates phosphorylation of Csp24 through at least two pathways: (1) by inhibition of the Rho/ROCK pathway, and (2) by an increase in activity of the cyclin-dependent kinase 5 (cdk 5) pathway. Another protein that is upregulated by paired CS-US training is calexcitin, a GTP- and Ca^{2+} -binding protein. Calexcitin is activated by PKC and has the dual role of inactivating the K^+ currents I_A and $I_{\text{Ca-K}}$ and binding to the Ryr receptor to release Ca^{2+} from internal stores.

Hermisenda is a model system that has provided a wealth of information about the cellular and molecular mechanisms underlying the convergence of CS and US pathways. What is missing now are many of the links between the various players, for example, the specific role of MAPK in the induction of LTM, the interaction between PKC activation, the role of 5-HT, and the function of the morphological changes for the expression of LTM. Recent work is now focusing on the role of the next order of cells, the effects of classical conditioning in the first level of interneurons. It will be of great interest to learn how the CS and US interact at these levels.

Lymnaea

Several forms of associative conditioning have been described in *Lymnaea*. These include operant conditioning of the respiratory response and classical conditioning of feeding behavior. The respiratory system in particular has the advantage of having a simple circuit, providing the opportunity to examine the specific roles of individual, identified neurons in the formation of memory. Specifically, the CPG neuron RPed1 has been shown to be a site of memory formation and storage. Similarly, feeding behavior, although it is composed of a slightly more complex circuit, has also provided an opportunity to study the role of a specific neuron, the cerebral giant cell (CGC) in memory formation. The feeding behavior is of further interest because this simple circuit is capable of rather complex conditioning. The emphasis of research in *Lymnaea* conditioning has been on the training parameters required for memory induction and on cell and circuit properties. Nevertheless, progress is also being made in the molecular mechanisms underlying cellular changes following conditioning.

Classical Conditioning

The *Lymnaea* feeding response can be classically conditioned, and both appetitive conditioning and conditioned taste aversion (CTA) have been demonstrated. By pairing either tactile stimulation (lip touch) or chemosensory stimulation (amyl acetate)

as the CS with sucrose (US), the CS gains the ability to elicit a feeding response. Appetitive conditioning can be induced with either multiple pairings or with a single pairing in animals that have been deprived of food for several days. To induce CTA, sucrose (CS) is paired with KCl (US). KCl alone decreases the feeding response and elicits a withdrawal response, and after pairing with sucrose, sucrose alone evokes a reduced feeding response that can last up to 1 month.

Classical conditioning in *Lymnaea* shares several features with classical conditioning observed in mammals, including taste discrimination and second-order conditioning. To test taste discrimination, animals were first tested separately with two CS stimuli, sucrose and carrot juice. CTA was established by ten pairings of CS1 (either sucrose or carrot juice) and the US (KCl). After conditioning, animals showed CTA to CS1, but not to the other unpaired CS. Thus, the animals can discriminate between different appetitive stimuli. To test second-order conditioning, these same animals were then given CS2 paired 10 times with CS1. When the animals were then tested with the presentation of CS2 alone, they showed a reduction in their feeding response, demonstrating that they are capable of second-order conditioning.

Cellular and molecular mechanisms In contrast to the extensive investigation into the behavioral aspects of learning and memory in the *Lymnaea* feeding system, examination of the molecular mechanisms required for memory induction is only beginning to be explored. As is typical of most memory systems, LTM of appetitive conditioning requires both transcription and translation, and, as was shown in *Aplysia*, the signaling pathways include MAPK, CREB, and C/EBP. At the cellular level, initial studies have found that injecting cAMP into a CPG neuron involved in the gating of the feeding response (CGC) induces LTF of synapses between CGC and feeding MNs, and this facilitation is blocked by injection of CRE oligonucleotides. The IEG C/EBP has been cloned in *Lymnaea*, and the level of C/EBP is modulated in MN B2 by CTA training. Training decreased LymC/EBP mRNA levels but increased translation and phosphorylation of LymC/EBP protein. Consolidation of LTM in *Lymnaea* appears to require nitric oxide (NO) signaling. Two different neuronal NO synthase genes have been cloned from *Lymnaea*, one of which shows a transient increase in mRNA levels after conditioning.

Operant Conditioning

Lymnaea have the capability to breathe through two different mechanisms. They can either absorb oxygen from the water directly through their skin by

cutaneous respiration, or, under anoxic conditions, they will come to the surface and breathe air into their lungs by opening a respiratory structure called a pneumostome. In the laboratory, anoxic conditions can be introduced by bubbling N₂ into a beaker of water. Snails can be operantly conditioned to reduce the number of times they open their pneumostomes for respiration by applying an aversive tactile stimulus to the lip area of the pneumostome whenever it opens.

The pattern of training trials (trial number and spacing) affects the duration of the memory induced. In general, two training trials (10–45 min each) at an ITI of 10 min to 1 h induce a protein synthesis-dependent form of memory (ITM) that lasts up to 2–3 h. LTM, defined by a translational and transcriptional dependence, is induced by increasing the number of training trials, the ITI, or both. Depending on the precise pattern of induction, LTM can last from up to 24 h to several weeks.

Maintenance of LTM in *Lymnaea* can be manipulated by extinction training, and by disruption of protein synthesis during reactivation of the memory. Three 45 min training trials given over 2 days induce LTM that lasts up to 5 days. Extinction trials (three sessions over 2 days, without reinforcement), begun 1 h after the original training, lead to an increase in pneumostome openings within 2 h. When tested 24 h after extinction training, spontaneous recovery of the operant behavior is observed.

As in *Hermisenda*, memory in *Lymnaea* also exhibits a protein synthesis dependence during memory reactivation. In *Lymnaea* reconsolidation experiments, LTM lasting 7 days was induced in one group of animals using training trials spaced over 2 days. A second group of animals served as yoked controls. On day 7, a single training session was given to all animals. Four hours later, animals that received contingent reinforcement showed memory, whereas yoked controls did not. Reactivation-induced memory was blocked if animals were cooled for 1 h immediately after reactivation or were injected with actinomycin to block transcription.

Cellular and molecular mechanisms Much of the recent research in *Lymnaea* operant conditioning has focused on the role of an identified neuron of the respiratory CPG, RPeD1. The CPG circuit responsible for respiratory pumping consists of a three interneurons (RPeD1, VD4, and IP3I) that are connected by reciprocal excitatory and inhibitory synapses. These neurons and their connections can be studied in semi-intact preparations as well as in isolated cell culture. A decrease in activity in RPeD1 is important for the induction of LTM. The intrinsic membrane

properties of RPeD1 are not altered by conditioning; however, RPeD1 neurons were more often quiescent in ganglia from trained animals, and their synapses onto IP3 were weaker. Operant conditioning was also found to affect the synaptic connections between CPG neurons and between RPeD1 and the pneumostome muscles. Operant conditioning reduced the number of IP3-induced RPeD1 bursts and reduced the percentage of cases in which RPeD1 stimulation induced pneumostome closure. In addition, in a gain-of-function experiment, it was found that although 2×20 min training sessions do not normally induce 18 h LTM, this training regimen paired with hyperpolarization of RPeD1 during the intervening ITIs did induce LTM. A more direct role for RPeD1 was found by Scheibenstock et al. When these authors ablated the soma of RPeD1 by intracellular injection of pronase, (1) the animal still showed normal respiratory behavior 2 days later, (2) RPeD1 maintained appropriate synapses with the other CPG cells, and (3) conditioning still induced ITM; however, LTM was not induced. If ablation of RPeD1 was delayed until 1 h after training, LTM was induced, indicating that RPeD1 is a site of induction for long-term operant conditioning in *Lymnaea*. The role of RPeD1 has also been studied in the induction of extinction and reconsolidation. Ablation of RPeD1 either 1 h after operant conditioning or 2 days before extinction training blocks extinction, and ablation immediately after reactivation blocks reconsolidation.

The signaling pathways involved in operant conditioning in *Lymnaea* have not been fully examined. CREB1 and CREB2 have been cloned in *Lymnaea*. *In situ* hybridization with a CREB2 antisense reveals the presence of CREB2 throughout the central nervous system (CNS), while similar labeling with a CREB1 antisense indicates that a much smaller population of cells expresses this mRNA. Neurons that express CREB1 include the CGC which is involved in the feeding system and RPeD1. Thus, the stage is now set for the investigation of these transcription factors, which have been clearly implicated in LTM in other systems.

Lymnaea provides an important system for the study of learning and memory because both the feeding and respiratory systems exhibit rather complex learning, especially considering the simplicity of the CPG circuits involved in the behaviors. It will be of considerable interest to map the signaling pathways and the synaptic changes responsible for these forms of memory.

Concluding Remarks

In conclusion, several molluscan preparations have contributed importantly to our understanding of the

cellular and molecular mechanisms of learning and memory. Each animal we have discussed exhibits very different types of memory in a variety of different response systems. Nonetheless, several striking similarities emerge. First, each system exhibits memories in three mechanistically distinct temporal domains. Second, many similar signaling cascades are implicated in memory formation, especially, PKA, PKC, and MAPK. Finally, LTM in each animal involves gene activation. Even more striking is the fact that these same temporal phases, signaling cascades, and transcriptional mechanisms are involved in a variety of forms of memory in higher mammalian species. Taken collectively, it appears that evolution has used a common tool kit to implement similar memory mechanisms across the animal kingdom.

See also: Invertebrate Models to Study Learning and Memory: *Lymnaea*; Learning and Memory in Invertebrates: *Aplysia*; Learning and Memory in Invertebrates: *Hermisenda*; Neuroplasticity: Computational Approaches; Synaptic Plasticity: Short-Term Mechanisms.

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