Cellular and Molecular Aspects of Short-Term and Long-Term Memory from Molluscan Systems

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Summary

Cellular and molecular mechanisms of short-term memory and long-term memory are reviewed based on observations of molluscan models of *Aplysia californica, Lymnaea stagnalis*, and *Hermissenda crassicornis*. It is generally accepted that short-term memory results from changes in the synaptic strength of preexisting neuronal connections that involve covalent modifications of preexisting proteins by various kinases. On the other hand, the synaptic plasticity underlying long-term memory is believed to involve protein synthesis and modulation of gene expression to induce new mRNA, protein synthesis, and morphologic modifications. These processes and mechanisms are compared in three molluscan model systems and likely have commonalities with those of mammals.

Key words Short-term memory, Long-term memory, Synaptic strength, Protein synthesis, Gene expression

Introduction

Although recent progress in molecular biology enables us to manipulate genes in mammals for a better understanding of higher brain function, molluscan models are still useful for studying the underlying mechanisms of brain function. It is difficult to study how synaptic plasticity produces a change in behavior in mammalian preparations owing to the large number of neurons and synapses involved in producing the behavior. Gastropod molluscs are established animal models for studying the neuronal mechanisms of learning and memory because the synaptic plasticity underlying changes in their behavior are easily observed.

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This chapter is dedicated to the late Professor Herman T. Epstein.

The mammalian brain has two memory systems: One is declarative, and the other is nondeclarative. Declarative memory is sometimes referred to as "explicit memory," or the conscious recall of knowledge, which is well developed in mammals and is dependent on cerebral cortical structures, including the hippocampus. In contrast, nondeclarative memory, sometimes referred to as "implicit memory," is memory for motor skills and involves the cerebellum and striatum in the mammalian brain. The learning mechanism studied in invertebrate models corresponds to that of nondeclarative memory.

In the mammalian brain, cellular and molecular changes that occur during the formation of both types of memory, declarative and nondeclarative, are difficult to study because the contribution of various synapses is not clearly identified. The invertebrate model system is useful for bridging this gap because the cellular and molecular analyses of behavioral problems using this simpler system facilitates our understanding of the synaptic loci and underlying fundamental mechanisms of learning and memory in general. The model animals exhibit several forms of learning—habituation, sensitization, classical conditioning, operant conditioning—which include many of the behavioral features of learning in mammals, suggesting that learning in molluscs and mammals has common mechanisms.

The modern physiology of learning and memory began during the early twentieth century with Pavlov's pioneering studies [1]. Canadian psychologist Donald Hebb published a theory of brain function and learning during the mid-twentieth century; it was a modified switchboard theory. A given memory was represented by a set of neurons that had developed increased functional connections. The basic idea was that new learning is fragile, and well-established memories are not; this process of establishing long-lasting memories is termed *consolidation*. Hebb proposed that if a neural connection interacted in a certain manner lasting cellular change would occur and an association would be formed, thereby providing the substrate of memory.

> When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.—Hebb [2]

Short-Term Memory, Long-Term Memory

This chapter focuses mainly on the cellular and molecular mechanisms of short-term memory (STM) and long-term memory (LTM) based on recent studies of gastropod molluscs, such as *Aplysia, Lymnaea*, and *Hermissenda*.

It is usually thought that STM lasts for minutes, whereas LTM lasts for days, weeks, years, or even as long as the entire life-span. There is also an intermediate-term memory (ITM) that falls between STM and LTM and lasts for ≥1 h. The cellular and molecular aspects of STM suggest that STM is due to a change in the synaptic strength of preexisting neuronal connections through covalent modification of preexisting proteins by various kinases such as protein kinase A (PKA),

protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMK II), and mitogen-activated protein kinase (MAPK). LTM, in contrast, is thought to involve the modulation of gene expression to induce new mRNA and protein synthesis. It is hypothesized that ITM requires new protein synthesis from preexisting transcriptional factors but does not require new protein synthesis from new mRNA synthesis, as is the case for LTM. ITM is sometimes referred to as the transition state from STM to LTM because although genetic translation is required genetic transcription is not [3, 4]. At the ITM to LTM stage, new structural alterations at the synapse are observed, such as the growth of new synaptic connections or synaptic remodeling. To analyze dynamic changes at the identified synapse, a gastropod model system is desirable and must be successfully conditioned with one-trial conditioning for in vivo study or must be observable in a network made up of dissociated cell cultures for in vitro study. The learned behavior must be such that there is a distinct time window of how long the memory persists. Our research strategy is to identify the time window of the transition from STM to LTM and then examine the effects of blocking genetic translation and transcription from the viewpoint of biophysics, morphology, and molecular biology.

The learning ability of each individual animal is quite different; some animals learn quickly and have good long-lasting memory even if they experience only one conditioning trial, whereas others have poor learning and memory performance even after several conditioning trials. These interindividual differences are thought to be due to individual differences in motivation that are dependent on the activity of the amygdala in mammals; however, these motivational differences exist even in invertebrates that lack a brain. In invertebrates, the physical condition, such as the state of starvation, influences their motivation, especially in feeding behavior-related conditioning paradigms. The differences between good and poor performers in terms of the conditioning paradigm and motivation are discussed below.

Aplysia californica

Aplysia is a well-established animal model for studying the synaptic plasticity underlying the gill- and siphon-withdrawal reflex. With this reflex, the animal shows several forms of learning, such as habituation, sensitization, and classical conditioning [5, 6]. The synapse between the sensory neuron and the motor neuron is the site of plasticity. Behavior in response to molecular mechanisms has been studied in isolated, semi-intact, and dissociated cell culture preparations. The molecular mechanisms contributing to implicit memory storage have been most extensively studied with the *Aplysia* gill- and siphon-withdrawal reflex [7].

Aplysia has a simple nervous system containing approximately 20 000 neurons and displays a variety of defensive reflexes for withdrawing its tail, gill, and siphon. A light touch to the siphon elicits withdrawal of both the siphon and gill, whereas a tactile stimulus to the tail elicits only tail withdrawal. These reflex withdrawals habituate with repeated stimulation. In response to a newly encountered stimulus to the siphon, the sensory neuron innervating the siphon generates excitatory

synaptic potentials in the interneurons and motor neurons. These synaptic potentials integrate spatiotemporally and strongly excite the motor neurons, leading to strong withdrawal of the gill. If the stimulus is presented repeatedly, the synaptic potentials produced by the sensory neurons in the interneurons and motoneurons become progressively smaller. The synaptic potentials in the motor neurons produced by some of the excitatory interneurons also become weaker, which results in a reduction in the strength of the reflex response. This reduction in the reflex response is termed *habituation* [8]. The decreased synaptic transmission in the sensory neurons results from a decrease in the amount of a chemical neurotransmitter, in this case glutamate, released from the synaptic terminals. When an animal repeatedly encounters a harmless stimulus it learns to habituate to the stimulus, whereas when the animal is exposed to a harmful stimulus it learns to respond more vigorously not only to the stimulus but also to other harmless stimuli. This reflex enhancement is termed *sensitization*. Sensitization is an elementary form of nonassociative learning by which *Aplysia* acquires information about the properties of a single noxious stimulus. As with other forms of defensive behavior, the memory for sensitization of the withdrawal reflex is graded, and repeated tail shocks lead to a long-lasting memory. A single tail shock produces short-term sensitization that lasts for minutes, and repeated tail shock produces long-term sensitization that lasts for up to 1 week [9]. Figure 1a shows a simplified diagram of habituation, and Fig. 1b shows a circuit diagram of short-term sensitization in *Aplysia*.

Sensory neurons in the abdominal ganglion innervating the siphon skin use glutamate as the neurotransmitter and terminate on motor neurons that innervate the gill. Stimuli to the tail activate sensory neurons that excite facilitating interneurons. Serotonin (5-hydroxytryptamine, or 5-HT) is the neurotransmitter of the facilitating interneuron, which forms a synapse on the terminal of the sensory neuron innervating the siphon skin and motor neuron controlling gill withdrawal (Fig. 1A). Habituation leads to homosynaptic depression, a decrease in synaptic strength resulting from sustained direct activity in the sensory neuron. On the other hand, sensitization involves heterosynaptic facilitation; that is, the sensitizing stimulus activates a group of interneurons that form synapses on the sensory neurons. Because the mechanism of sensitization in *Aplysia* has been well studied and is described in detail elsewhere (e.g., in a textbook [10] and in reviews [7, 11–13]), only a brief summary of the in vivo and in vitro systems is provided here.

In a dissociated culture network of sensory and motor neurons, a brief application of 5-HT, a modulatory transmitter normally released from the facilitating interneuron by sensitizing stimuli in the intact animal, mimics a tail shock, leading to short-term facilitation of the motor neurons [14]. If 5-HT is applied intermittently at some interval, however, it induces long-term facilitation [15]. Furthermore, if the sensory neurons as presynaptic elements are in an excitatory state before the 5-HT is applied, the facilitating effect induced by repeated 5-HT application is larger and longer lasting. This phenomenon is the same as classical conditioning [16]. Serotonin binds to cell surface receptors on the sensory neurons and facilitates the production of a diffusible second messenger, cyclic adenosine monophosphate (cAMP), by activating adenylyl cyclase. The increase in cytosolic cAMP results in

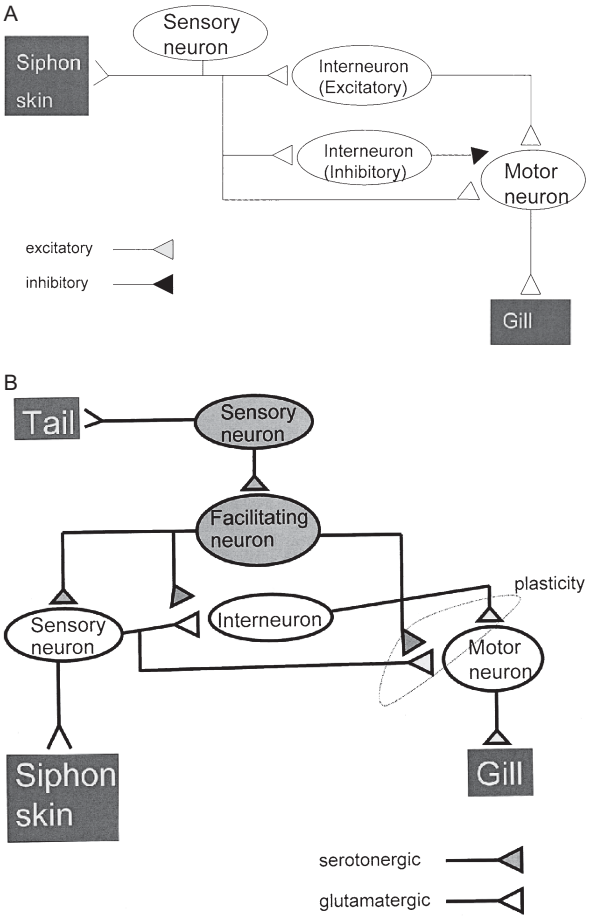

Fig. 1. **A** Simplified circuit involved in the gill-withdrawal reflex. In this circuit, mechanosensory neurons innervate the siphon skin. These sensory cells use glutamate as their neurotransmitter and terminate on a cluster of six motor neurons that innervate the gill and on several groups of excitatory and inhibitory interneurons that synapse on the motor neurons. Repeated stimulation of the siphon leads to depression of synaptic transmission between the sensory and motor neurons as well as between certain interneurons and motor neurons. **B** Sensitization of the gill is produced by applying a noxious stimulus to another part of the body. Stimuli to the tail activate sensory neurons that excite facilitating interneurons. The facilitation cells, some of which use serotonin (5-hydroxytryptamine, or 5-HT) as their neurotransmitter, form synapses on the terminal of the sensory neurons innervating the siphon skin. There they enhance transmitter release from the sensory neurons by means of presynaptic facilitation

short-term facilitation. This facilitation is due to enhancing the release of the neurotransmitter from the sensory receptor terminal, thus inactivating K^+ channels [9], and enhanced excitability. All of the mechanisms mentioned above are presynaptic events; that is, they occur in the sensory neuron. At the sensory-to-motor neuron synapse, the degree of sensitization seems to depend on both the duration of 5-HT exposure and the state of the synapse—that is, whether the synapse is in a resting or a depressed state. Many studies have demonstrated that brief exposure to 5-HT activates PKA in the sensory neuron, leading to inactivation of K^+ channels, an increase in intracellular Ca^{2+}, and transmitter release [17–19]. Longer exposure to 5-HT activates PKC and CaMK II in the sensory neuron [20, 21]. In addition, longer exposure to 5-HT leads to intermediate-term facilitation, which requires protein synthesis [20]. Exposure to 5-HT for more than 5 min might involve postsynaptic mechanisms such as inositol trisphosphate-mediated CaMK II activation and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor insertion [22, 23], as well as presynaptic mechanisms such as simultaneous PKA and PKC activation. These findings suggest that not only the specific kinase involved but its site of action might depend on the duration of transmitter exposure, in this case 5-HT; brief exposure affects the presynaptic mechanism, whereas long exposure involves both presynaptic and postsynaptic mechanisms.

Activity-dependent plasticity in *Aplysia* might involve both pre- and postsynaptic mechanisms. At the synapse between sensory and motor neurons, presynaptic tetanic stimulation evokes long-term potentiation similar to that observed in the mammalian hippocampus. This potentiation involves metabotropic glutamate receptors, and potentiation decreases after injection of Ca^{2+} into the sensory neuron or injection of a CaMK II inhibitor into the motor neuron [23, 24]. These findings indicate that potentiation involves both pre- and postsynaptic mechanisms that interact with each other (i.e., with a strong shock to the tail both postsynaptic Ca^{2+} and CaMK II and presynaptic PKA contribute to sensitization, whereas sensitization with a weak shock is an entirely presynaptic event). Classical conditioning experiments in *Aplysia* with a siphon touch [conditioned stimulus (CS)] and tail shock [unconditioned stimulus (US)] revealed that both pre- and postsynaptic mechanisms are involved [25].

Briefly, with STM, synaptic stimulation of sensory neurons leads to a local increase in cAMP and activation of PKA by causing the catalytic subunits of the enzyme to dissociate from the regulatory subunits. The catalytic subunits then phosphorylate K^+ channels and proteins to enhance transmitter release. In contrast, repeated synaptic stimulation resulting in a persistent increase of cAMP levels leads to long-term synaptic plasticity.

In addition to activating various protein kinases after STM, protein phosphatases such as calcineurin and protein phosphatase 1, acting as inhibitory constraints of memory formation, are also suggested to have a key role in regulating LTM [26]. It is generally assumed that an equilibrium state between kinase and phosphatase activities at a given synapse is critical to gate the synaptic signal reaching the nucleus to stabilize memory formation and retrieval. LTM is represented at the cellular level by activity-dependent modulation of both the function and structure

of specific synaptic connections, which in turn depend on activation of a specific pattern of gene expression [7]. PKA activates gene expression by phosphorylating transcription factors that bind to the cAMP-responsive element (CRE). The CRE is one of the DNA response elements contained in the control region of the gene. The binding of various transcription factors to these response elements regulates the activity of RNA polymerase, thereby determining when and to what level a gene is expressed. One of the major factors that recognize the CRE is a CRE-binding protein (CREB1), a transcriptional activator. Dash et al. demonstrated that PKA activates gene expression through CREB during the formation of LTM. If CREB1 is essential for LTM, selective blockade of CREB1 should eliminate LTM formation. A CRE oligonucleotide injected into a sensory neuron co-cultured with motor neurons inhibits the function of CREB1 by binding to the CREB1 protein in the cell [27]. Various transcriptional enzymes of the CREB family are involved in LTM formation; CREB1 acts as an activator, and CREB2 acts as a repressor. It seems that a balance between the CREB activator and repressor is important for LTM formation. Overexpression of an inhibitory form blocks LTM but not STM, whereas overexpression of an activator has the opposite effect and increases the efficacy of training in LTM formation.

After the LTM-related structural modification occurs at the presynaptic sensory neuron varicosities due to sensitization, sensory neurons exhibit a twofold increase in the total number of synaptic varicosities and in the size of each neuron's arbor [28–30]. Bailey and Chen reported that, after behavioral extinction of sensitization, changes in the varicosities and active zone number persisted for at least 1 week and were partially reversed by the end of the 3-week experiment [30]. Kim et al. observed functional and presynaptic structural changes during long-term facilitation with time-lapse confocal microscopic imaging. Long-term facilitation results in structural changes in presynaptic neurons. These findings suggest two possible mechanisms: activation of preexisting silent presynapses through filling with synaptic vesicles or the generation of new synaptic varicosities. The activation of preexisting silent presynapses, a rapid process that occurs within 3–6 h after facilitation, requires only translation, whereas the generation of new varicosities is a comparatively slow process that occurs within 24 h and requires both translation and transcription [31].

Lymnaea stagnalis

A number of classic conditioning [32–36] and operant conditioning [37, 38] paradigms have been used successfully in *Lymnaea*, and cellular traces of behavioral conditioning have been identified in isolated, semi-intact, and simplified culture networks. In this model system, associative learning of appetitive or aversive conditioning of the feeding behavior and aversive operant conditioning of respiratory behavior are well characterized. To understand the conditioning-induced modification of the neural networks underlying feeding behaviors, four groups of neurons—sensory, modulatory, central pattern generator, and motor neurons—have been

studied, and the components of the neurons in each group have been analyzed at the cellular and molecular levels.

Previous studies examined the chemosensory neuron network—cerebral giant cells (CGC), slow oscillator cells (SO), and cerebral ventral 1 cells (CVl) in the modulatory neuron group; N1, N2, and N3 in the central pattern generator neuron group; and B7, B3, and B4 in the motor neuron group—leading to sequences of muscle activity and feeding movements [39, 40] for appetitive conditioning. It is hypothesized that CGC and B2 motor neurons have key roles in mediating aversive conditioning [34, 41]. Three neuron groups are involved in the aversive operant conditioning of respiratory behavior: sensory neurons, respiratory central pattern generator, and motoneurons controlling the pneumostome muscle. Sensory neurons located in the pneumostome-osphradial area activate the respiratory central pattern generator, comprised of the right pedal dorsal 1 (RPeDl), visceral dorsal 4 (VD4), and input 3 (IP3) neurons. VD4 drives activity in the K motoneurons responsible for pneumostome closing, and IP3 drives activity in the I/J motoneurons responsible for pneumostome opening [42]. Studies of this operant conditioning preparation indicate that RPeD1 is the necessary site for LTM formation and memory storage because following soma ablation the neural circuit is capable only of mediating learning and ITM. LTM cannot be demonstrated after soma ablation. Thus, the soma, where the new mRNA is synthesized to make new protein for LTM formation, is the necessary site as it functions as a protein-synthesizing factory [4, 42, 43]. Figure 2 shows circuit diagrams and neurons involved in feeding behavior and respiratory behavior in *Lymnaea*.

In the appetitive conditioning paradigm, light mechanical touch around the lip is a CS, and sucrose is a US [39]. For aversive conditioning, sucrose is a CS, and aversive KCl application or moderate mechanical touch to the head acts as a US [35, 44]. Both one-trial appetitive and aversive conditioning have been successfully performed, resulting in the formation of LTM [45–49]. A single appetitive conditioning trial results in memory that persists at least 21 days [46], and the memory of a single aversive conditioning trial persists at least 24 h to 7 days [49]. It is interesting that the motivation of the animals differs between the appetitive and aversive conditioning paradigms [49, 50]. For appetitive conditioning, the animals were food-deprived for 4 days prior to the conditioning, and this condition was sufficient for good performance [46, 50]. In contrast, food deprivation for 5 days did not motivate animals, as indicated by good retention; rather, CS-sucrose causes cessation of feeding behavior in more than half of the animals tested [49]. It is unlikely that 5 days of food deprivation made the animals sick; instead, a long period of food deprivation might have induced excessive stress, which decreased their motivation.

It is generally assumed that there are two critical periods for LTM formation and for memory recall; one is activated soon after the conditioning trial to synthesize protein from preexisting transcriptional factors, and the other is activated later to synthesize protein from new transcriptional factors. In accordance with this hypothesis, injection of the translation blocker anisomycin 2.5 h before training prevents the formation of ITM (lasting 1–3 h) and LTM (lasting > 6 h). On the other

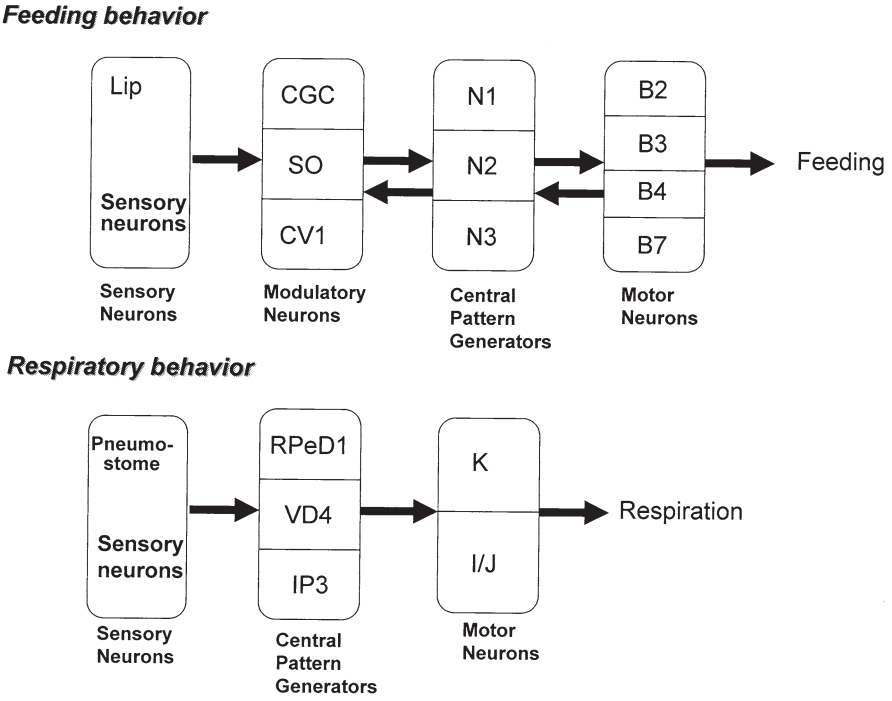

Fig. 2. Feeding and respiratory networks of *Lymnaea stagnalis*. **Top panel** Neuronal network underlying feeding behavior. Chemosensory neurons located in the lip detect the presence of food. The sensory neurons send their information to modulatory neuron groups: cerebral giant cells (*CGC*), slow oscillator cells (*SO*), and cerebral ventral 1 cells (*CV1*). The rhythmic pattern of the central pattern generators produces the feeding cycle of protraction, rasp, and swallow phase, leading to muscular activity of B2, B3, B4, and B7 motor neurons. **Bottom panel** Neuronal network underlying respiratory behavior. Activation of sensory neurons located in the pneumostome-osphradial area leads to activation of the respiratory central pattern generators comprising right pedal dorsal 1 (*RPeD1*), ventral dorsal 4 (*VD4*), and input 3 (*IP3*) neurons. VD4 drives activity in K motoneurons, and IP3 drives activity in I/J motoneurons, which are responsible for pneumostome closing and opening, respectively

hand, injection of the transcription blocker actinomycin-D 2.5 h before training did not prevent the establishment of ITM but blocked LTM formation. Thus, in *Lymnaea*, following aversive operant conditioning both ITM and LTM are dependent on new protein synthesis [4]. In addition to pharmacologic blockade using anisomycin and actinomycin D, translation and transcription factors can be physically manipulated. Immediately after "taste avoidance conditioning" in *Limax*, Sekiguchi et al. exposed animals to 1°C for 1 h to induce retrograde amnesia [51]. Using this cooling technique in the respiratory operant conditioning of *Lymnaea*, Sangha et al. reported that cooling the animals for 1 h immediately after training was sufficient to block both ITM and LTM, whereas cooling them for a similar

period starting 10 or 15 min after cessation of training failed to block ITM or LTM formation, respectively [52]. Further cooling extended LTM that normally persisted for 2 days to at least 8 days [52], demonstrating that cooling prevents forgetting, and thus forgetting is an active process that is not part of the memory consolidation process. This operant conditioning can be extinguished with the spaced backward conditioning procedure more effectively than with massed conditioning trials. The memory was extinguished within 1 h after the extinction conditioning trial; the extinction was due to new mRNA and protein synthesis at the soma of the RPeDl, which is required for LTM consolidation [53]. A series of experiments by Sangha et al. demonstrated that there are two critical periods required for LTM: one immediately after conditioning and the other several hours later. This two-stage protein synthesis theory, however, was not supported in the one-trial appetitive conditioning paradigm [45].

Pharmacological blockade by injection 10 min after conditioning of either the translation inhibitor anisomycin or the transcription inhibitor actinomycin-D blocked LTM. Further anisomycin injection 1, 2, 3, 4, 5, and 6 h after the conditioning paradigm had no effect on memory recall. These results are in contrast to those from the aversive operant conditioning mentioned above and indicate that there is only a single critical period between 10 min and 1 h for protein synthesis in appetitive conditioning. These differences might reflect the involvement of a different neuronal network in each conditioning paradigm. Consolidation is believed to involve the regulation of genes involved in long-term stabilization of synaptic modifications in the neuronal circuits activated during learning.

A critical step in this process involves the activation of immediate early genes (IEGs), which are rapidly induced activity-dependent genes that encode transcription factors capable of regulating the transcription of a number of downstream late-responding genes. It is generally assumed that the crucial point for memory consolidation is the timing of IEG activation. Several IEGs—such as CCAAT/enhancer-binding protein (C/EBP), activity-related cytoskeleton associated protein (Arc), c-fos, and c-jun—have been studied with regard to the stabilization of long-lasting synaptic plasticity and LTM formation [54–58]. Recent findings indicate that LTM formation in aversive conditioning for feeding behavior involves a combination of C/EBP synthesis and phosphorylation as well as C/EBP mRNA breakdown in the pair of B2 motoneurons that control feeding behavior [59].

Hermissenda crassicornis

Hermissenda is one preparation that has contributed to an understanding of Pavlovian classical conditioning of visual (CS) and vestibular (US) turbulence at the cellular and molecular levels [60–63]. This animal can learn the sequential event of light and vestibular turbulence. Naive *Hermissenda* exhibit positive phototoxic behavior; that is, they move forward in response to light and contract their foot in response to turbulence—whereas after paired presentations of light and orbital rotation they hesitate to move toward light. Animals receiving the same amount of

stimuli without overlapping the CS and US (i.e., pseudo-random conditioning) or light and/or rotation alone do not show any conditioned behavior in response to the light stimulus. This demonstrates that only the paired presentation of the CS and US results in associative learning. The conditioned behavior is evaluated by observing the foot length; naive animals extend their foot, whereas conditioned animals contract their foot in response to the light stimulus.

The central nervous system in *Hermissenda* is relatively simple, making it possible to identify the neurons in the neuronal circuits that are involved in the conditioning. The two sensory systems mediating the CS and US have been described in detail by Alkon and colleagues [64–68]. In addition, the convergent site providing the synaptic interaction between the CS and US has been identified [66, 69–72]. The CS is detected by a pair of eyes comprising five photoreceptor cells, which are subdivided into two types in terms of sensitivity to light: two type A photoreceptors and three type B photoreceptor cells. The vestibular sensing organ is a pair of statocysts comprising 13 hair cells. The caudal hair cells synapse onto the medial type B photoreceptor via a γ-aminobutyric acid (GABA)ergic synapse [73–75]. This synapse is the primary locus of plasticity after the conditioning. Because the medial type B photoreceptor is not only a photoreceptor but also a postsynaptic neuronal element, many studies have focused on this synapse to elucidate the mechanisms of classical conditioning from the viewpoint of biophysics, biochemistry, morphology, and molecular biology.

Two sensory receptors, photoreceptors and statocyst hair cells, mediating the CS and the US, respectively, are located in both the cerebral and pedal ganglia of the circumesophageal nervous system, and thus their synaptic projections remain intact even in the isolated brain preparation. Thus, this unique model system allows us to study in vitro conditioning employing the natural CS and US stimuli used for in vivo studies [76–78]. The mechanism of CS–US contiguity has been identified and has been the focus of neuroinformatic, biophysical, biochemical, morphologic, and molecular biologic analyses [79, 80]. Figure 3 shows a simplified circuit diagram involving the visuovestibular associative learning in *Hermissenda*.

Squire and Alvarez defined memory consolidation as “the molecular cascade and morphologic changes whereby synaptic modifications gradually become stable after learning” [81]. This definition also applies to invertebrate models of consolidated long-term memory (CLTM). The relation between the number of training events (TEs), defined by the number of paired presentations of the CS and US, and the number of retention days was evaluated from behavioral observations made by Epstein and colleagues showing the time window for the formation of STM to CLTM [82–84]. The STM lasts 7 min with one or two TEs. Nine TEs result in LTM within 60 min that lasts less than 1 day; and CLTM occurs 220 min after the conditioning and lasts for at least 6 days. STM requires no protein synthesis, whereas both LTM and CLTM require protein synthesis. Thus, the spectrum of events that correlate with the establishment of these memory stages and the transitions between them can be readily studied. The two-critical-periods theory of protein synthesis for LTM formation mentioned above has also been supported in this model system;

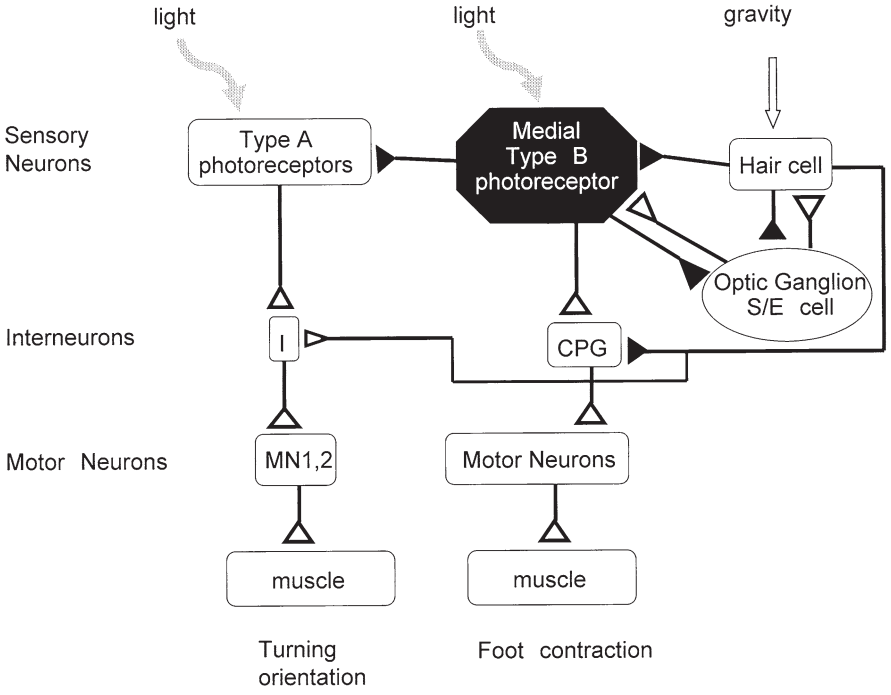

Fig. 3. Flow of visual and vestibular information in *Hermissenda crassicornis*. The conditioned stimulus is received at five photoreceptors, and the unconditioned stimulus is sensed by 13 hair cells. Photoreceptors are subdivided into two type A and three type B cells. The medial type B photoreceptor is not only a photosensitive neuron but also a postsynaptic neuron of statocyst hair cells. Thus, the primary conditioning effect is observed at the type B photoreceptor. Increased excitability of type B cells facilitates foot contraction. *Filled triangles*, inhibitory synapses; *open triangles*, excitatory synapses. *MN*, motor neuron; *CPG*, cerebropleural ganglion

the first critical period is up to 15 min, and the other is 60–220 min after the conditioning. The translation inhibitor anisomycin blocks memory recall when applied 13 min and 60–220 min after the conditioning. There is good memory recall from 10 to 60 min. It is assumed that the period from 15 to 60 min after the conditioning involves the activities of the proteins already made, but with no new protein synthesis [83].

Consistent with this result, morphologic modifications are observed at the axon terminal of the type B photoreceptor, which is the postsynaptic element for the vestibular hair cells and presumed to control whole-animal movement during phototaxis [78, 85]. Kawai et al. demonstrated that the entire volume of the terminal branch arborization of the type B photoreceptor axon starts to decrease significantly soon after several presentations of the CS and US, peaks at 10 min, and remains at the same elevated level for up to 60 min in an isolated preparation. This decrease was originally discovered in an in vivo preparation after acquisition of learning by

Alkon et al., who called it *focusing* [86]. During focusing, an increase in input resistance also occurs in the neurons of the in vitro preparation. The dynamic nature of the morphology and physiology is completely parallel for up to 60 min after the conditioning. Kawai et al. also demonstrated that no morphologic modification occurs in a pre-anisomycin-treated preparation [85]. The in vitro conditioning-induced synaptic focusing is also prevented by injecting the Ca^{2+} chelator BAPTA, the ryanodine receptor blocker dantrolene, or micromolar concentrations of ryanodine into the type B photoreceptor. These results indicate that morphologic modification after in vitro conditioning involves the ryanodine receptor [87].

The early stage of protein synthesis without mRNA modification is obvious in this preparation. The possible activities include interactions among existing substances in their various intracellular compartments. During the 60- to 220-min period, either anisomycin or actinomycin-D suppressed recall. This period is assumed to be the second period of protein synthesis. Cell adhesion molecules (CAMs) such as HNK-1, neural CAMs, and integrins have pivotal roles in long-term potentiation in both invertebrates and mammals [87–89]. The most common extracellular matrix protein tripeptide sequence, Arg-Gly-Asp (RGD), acts as a CAM-competitive inhibitor and can disrupt memory function. The latter period, when new dendritic spines and synapses are formed, is the time when CAM inhibitors are effective. Animals treated with RGD applied 10 min following conditioning exhibited complete inhibition of learning or no recall of the conditioned behavior. Animals treated with RGD applied 20–50 min after training, however, had a marked decline in inhibition [82]. LTM in *Hermissenda*, as well as transcription and translation inhibitor response criteria, is established within 60 min [83]. LTM consolidation requires 60–230 min [82, 83]. This interval is too long to be due to transmitting signals through synapses or new protein synthesis transported anterogradely from the soma.

Studies by Alkon et al. demonstrated the protein synthesis mechanism that is required for consolidating associative learning into LTM [90]. The application of a potent PKC activator, bryostatin, for 2 days before conditioning induces the synthesis of proteins that are necessary and sufficient for subsequent CLTM. Under normal conditions, two TEs with paired CS and US cause STM lasting 7 min; after a 4-h exposure to subnanomolar (0.1–0.25 ng/ml) concentrations of bryostatin on the 2 days preceding conditioning, however, the same two TEs produce CLTM that lasts longer than 1 week and is not blocked by anisomycin. Anisomycin, however, eliminates LTM lasting at least 1 week after nine TEs. Both the nine TEs alone and the two TEs with bryostatin exposure induce a comparable increase in the PKC α-isozyme substrate calexcitin in the type B photoreceptor, which shows a Pavlovian conditioning-dependent increase in phosphorylation and absolute quantity [91], and enhances PKC activity in the membrane fraction. The specific PKC antagonist Ro-32-0432 or anisomycin blocks bryostatin-induced protein synthesis as well as bryostatin-induced enhancement of behavioral conditioning [90]. Electrophysiologic measures of input resistance and long-lasting depolarization in response to a light stimulus also demonstrated that bryostatin induces excitability in the type B photoreceptor [92]. Bryostatin increases the synthesis of calexcitin in the type B

photoreceptor, as occurs with Pavlovian classical conditioning of *Hermissenda* [93]. Bryostatin in low doses (0.1–0.25 ng/ml) initially enhances PKC activation followed by down-regulation and then prolonged enhancement of protein synthesis. Bryostatin-induced PKC enhancement of protein synthesis enhances the duration of the memory of Pavlovian classical conditioned responses. Bryostatin-induced PKC activation on days before training is sufficient to cause LTM. This LTM does not require protein synthesis after the training. PKC, after activation induced by bryostatin, is down-regulated by two distinct pathways: one that is proteasome-mediated and another that is mediated by phosphatases such as protein phosphatase 1 and protein phosphatase 2A [94]. Higher bryostatin concentrations (≤1.0 ng/ml) block memory retention because PKC synthesis cannot compensate for inactivation and down-regulation; therefore, the available PKC is depleted and memory retention is blocked [90].

Lymnaea can be conditioned with the same CS and US paired presentation as *Hermissenda*, but the underlying mechanisms for visuovestibular associative learning are suggested to be different [36, 95].

Conclusion

Cellular and molecular mechanisms underlying STM and LTM are reviewed based on observations of molluscan models. STM results from changes in the synaptic strength of preexisting neuronal connections that involve covalent modifications of preexisting proteins by various kinases. The synaptic plasticity underlying LTM is believed to involve protein synthesis and modulation of gene expression to induce new mRNA, protein synthesis, and morphologic modifications. These processes and their mechanisms in the three molluscan model systems explored herein likely have commonalities with those of mammals.

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