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Non-synaptic neuronal mechanisms of learning and memory in gastropod molluscs

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1. ABSTRACT

Gastropod molluscs provide important model systems for investigating the behavioral and neural basis of associative and non-associative learning. Habituation, sensitization, classical and operant conditioning are studied in motor reflex and central pattern generator circuits. Although synaptic plasticity has long been recognized as playing a key role in molluscan learning circuits, non-synaptic changes resulting in alterations in the excitability of neurons are increasingly recognized as an essential component of the memory trace.

2. INTRODUCTION

It has long been recognized that changes in synaptic strength play a major role in molluscan learning and memory (1,2) but an increasing number of examples of non-synaptic plasticity have been discovered that form part of the memory mechanism in neural circuits underlying behavioral learning. These non-synaptic mechanisms include changes in input resistance, membrane potential and threshold for plateau initiation. Changes in these non-synaptic properties usually alter the excitability of the neurons (Figure 1A), with learning-induced changes in intrinsic ionic currents providing the underlying mechanism. This contrasts with synaptic plasticity where the strength of synaptic connections between neurons is changed after learning (Figure 1B).

3. CHANGES IN INPUT RESISTANCE

Examples of input resistance increases induced by conditioning occur in the photoreceptor cells of *Hermissenda* (3). Conditioning of phototactic behavior induces cellular changes in both the type A and type B photoreceptors, which increase the firing of the cells in response to light (the CS) compared with controls. The CS evokes a larger receptor potential and a persistent enhanced excitability in the B cells as tested by the response to a standard applied depolarizing pulse. An increase in membrane resistance contributes to this increase in spike activity and a decrease in spike accommodation is also important in producing a sustained response to receptor depolarization in response to the CS. Conditioning reduces the peak amplitude of several types of conductances in the B type photoreceptors including a calcium-dependent ($I_{K,Ca}$) and voltage-dependent currents ($I_{A,}$ I_{Ca}) (4-6). Importantly, these conductance changes can be mimicked by the application of 5-HT providing evidence that the increased release of this transmitter via the hair cell polysynaptic pathway might be responsible for the changes in the intrinsic properties of the photoreceptors following conditioning (reviewed in 3).

Several second messenger systems have been shown to be involved in induction of cellular plasticity in *Hermissenda* (reviewed in 3). Activation of the PKC and ERK signaling pathways both appear to contribute on the

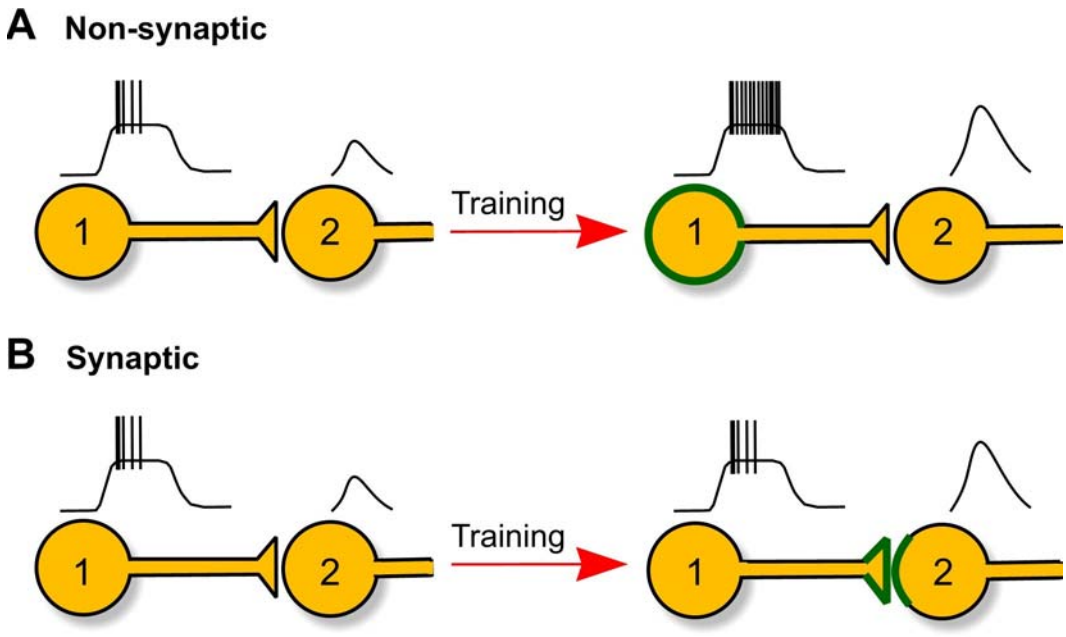

Figure 1. A schematic comparison of non-synaptic (A) and synaptic (B) mechanisms of training-induced plasticity. A. Cartoon showing an example of non-synaptic plasticity. As a result of training, the intrinsic excitability of the pre-synaptic neuron (1) increases, which leads to a higher rate of action potential firing in response to a depolarizing stimulus and causing an enhanced response in the post-synaptic neuron (2). B. Cartoon showing an example of synaptic plasticity. Training results in a strengthening of the synapse between the pre- and post-synaptic cell, which leads to an enhanced response in the post-synaptic cell even though the action potential firing rate of the pre-synaptic cell remains unchanged. Bold green outlines in A and B indicate sites of plastic changes that can be pre- and/or post-synaptic. These changes in neuronal excitability and synaptic strength are shown to occur the soma region of the neuron but in molluscs they are likely also to occur at neuritic loci.

modulation of the different types of K^+ channels in the type B photoreceptors. Blocking of PKC with kinase inhibitors prevents the induction of short-term excitability and reverses its maintenance in conditioned animals. PKC is thought to phosphorylate the $I_{K,A}$ and $I_{K,Ca}$ channels, decreasing their maximum conductance and producing increased input resistance and evoked spike frequency. Phosphorylation of ERK occurs after conditioning and application of 5-HT, the latter acting partly through a Ca^{2+}-dependent PKC pathway.

It was previously thought that changes in the excitability of the *Hermissenda* photoreceptors was the sole mechanism for associative conditioning (7) but the more recent identification of interneurons connecting the photoreceptors to motoneurons invoved in the behavioral response has identified further sites of plasticity involved in learning. For instance, the intrinsic excitability of the I_e interneurons also appears to be increased after conditioning at holding potentials more positive than -60mV (8).

Similar input resistance increases have been described in the LE mechanoreceptors of the gill-siphon reflex of *Aplysia* following classical conditioning (9). This non-synaptic mechanism increases the excitability of the sensory neurons, tested directly by current injection, making it more likely that the sensory neurons will respond to siphon touch after conditioning. The increase in excitability could be due to a decrease in a specific type of intrinsic potassium current (s-channel) that was identified when serotonin was applied to sensory neurons in the intact ganglion (10).

4. CHANGES IN MEMBRANE POTENTIAL

Persistent changes in membrane potential occur in whole body withdrawal interneurons in *Helix* (11) and in feeding command-like CV1 (cerebral ventral 1) interneurons in *Lymnaea* (12). In both snails the cells are depolarized following conditioning and this lowers the threshold for firing in response to the CS thus allowing command cells to directly activate the motor circuits. In *Helix*, there is an additional decrease in threshold for spike initiation in withdrawal interneurons (11) that also promotes the conditioned withdrawal reflex. In *Lymnaea*, a long-lasting membrane depolarization of 11 mV on average was recorded in the CV1 neurons from conditioned compared with control snails that persisted for as long as the electrophysiological and behavioral memory trace (up to 4 days). The depolarization makes the cells more responsive to the CS following tactile conditioning and can account for the activation of the feeding response via the CV1 cell's strong excitatory synaptic connection with interneurons of the feeding CPG (central pattern generator) (Figure 2A). The importance of this result is emphasized by experiments where the membrane potential of the CV1 cells are manipulated to either reverse the effect of behavioral conditioning or to mimic the effects of

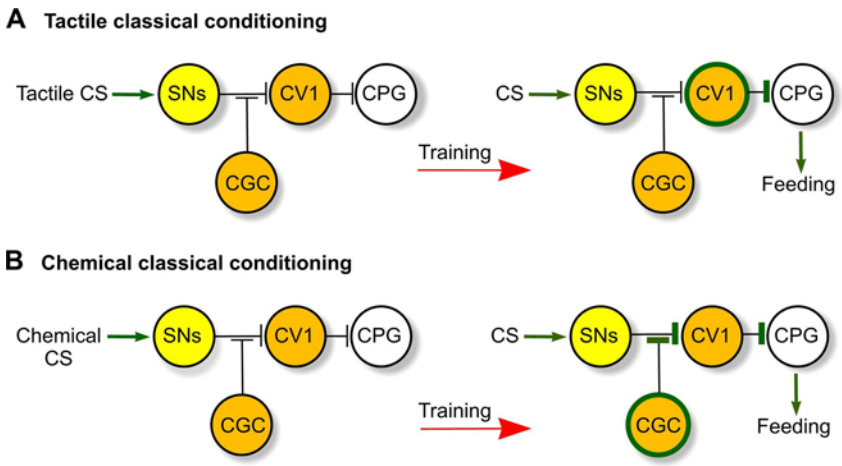

Figure 2. A comparison of the identified loci of non-synaptic changes in tactile (A) and chemical (B) and food-reward classical conditioning in *Lymnaea*. A. In tactile classical conditioning, the command-like CV1 cells become depolarized after training (bold green outline) resulting in an enhanced activation of these neurons by the CS and an increased synaptic output to the feeding CPG. B. In chemical classical conditioning, training leads to a persistent depolarization (bold green outline) of the extrinsic modulatory cell type CGC without affecting the membrane properties of the CV1s. Depolarization of the CGC however facilitates the connection between the chemosensory neurons and the CV1s resulting in an enhanced output from the latter to the feeding central pattern generator (CPG). Synapses with enhanced output after training are indicated with bold green bars. Abbreviations: CS, conditioned stimulus; SNs, sensory neurons; CGC, Cerebral Giant Cell; CPG, central pattern generator; CV1, cerebro-ventral 1.

conditioning in naïve snails (12). These experiments showed that the persistent depolarization of the CV1 cells was both sufficient and necessary for the conditioned tactile response in the feeding network.

The *Aplysia* homologues of the *Lymnaea* CV1 cells, the CBI-2 cell type, has been examined after *in vitro* classical conditioning of feeding but unlike *Lymnaea*, no changes in the membrane potential or other non-synaptic cellular properties of the CBIs were recorded (13).

The CGCs (Cerebral Giant Cells) in *Lymnaea* also show persistent depolarization of cell body membrane potential of about 10 mV compared with controls after one-trial chemical conditioning (14). This depolarization indirectly increases the strength of postsynaptic responses to stimulation by the chemical CS by a process that involves an increase in intracellular calcium concentration of the CGC proximal axonal processes (14). The local targets for CGC depolarisation are the CV1 command cells for feeding (Figure 2B) and artificial depolarization of the CGCs in naïve snails increases the response of the CV1 cells to the CS, mimicking the effects of behavioral conditioning. The CGCs are extrinsic to the feeding circuit and so the change of membrane potential activates feeding responses indirectly by affecting command interneurons intrinsic to the circuit. It appears that the CGCs are increasing the strength of the CS to CV1 synapse by presynaptic facilitation. This learning-related mechanism is independent of the normal 'gating' function of the CGCs that depends on their tonic firing (15). This firing rate is not affected by the learning-induced persistent depolarization of the CGCs (14), so the change in synaptic strength is mediated by a change in membrane potential alone. An example of a facilitation of synaptic transmission by cell body depolarization alone is shown in Figure 3. It cannot be due to any extrinsic factors like tonic synaptic input because the pre- and postsynaptic cells are recorded in cell culture.

A persistent sodium current present in the CGCs is a strong candidate for modulation following conditioning because it is involved in setting the long-term electrical properties of the cells. It has been shown (16) that it makes significant contribution to the membrane potential of the CGCs. Injection of cAMP into the CGCs increases the size of the persistent sodium current resulting in a long-term depolarization of the cells. The fact that this occurs in isolated CGCs indicates that the changes due to conditioning are likely to be intrinsic to these cells.

The onset of the CGC depolarization is between 16-24 hr after training and it persists for at least 14 days, as long as the behavioral memory trace was present. There is an early behavioral memory trace from 2 hr after conditioning so the CGCs cannot be involved in memory expression immediately after training. It is more likely that the CGCs are involved in the maintenance of the LTM after the trace has already been consolidated and encode information that is important for memory recall. Interestingly, the CGC's changes occur in chemically-conditioned snails (Figure 2B) but not those subjected to tactile conditioning. For tactile conditioning the CV1 cells are depolarized (Figure 2A) but not the CGCs (12). The reason for this difference in the two types of classical reward conditioning is unclear but is probably linked to differences in the neural pathways activated by amyl acetate versus lip touch.

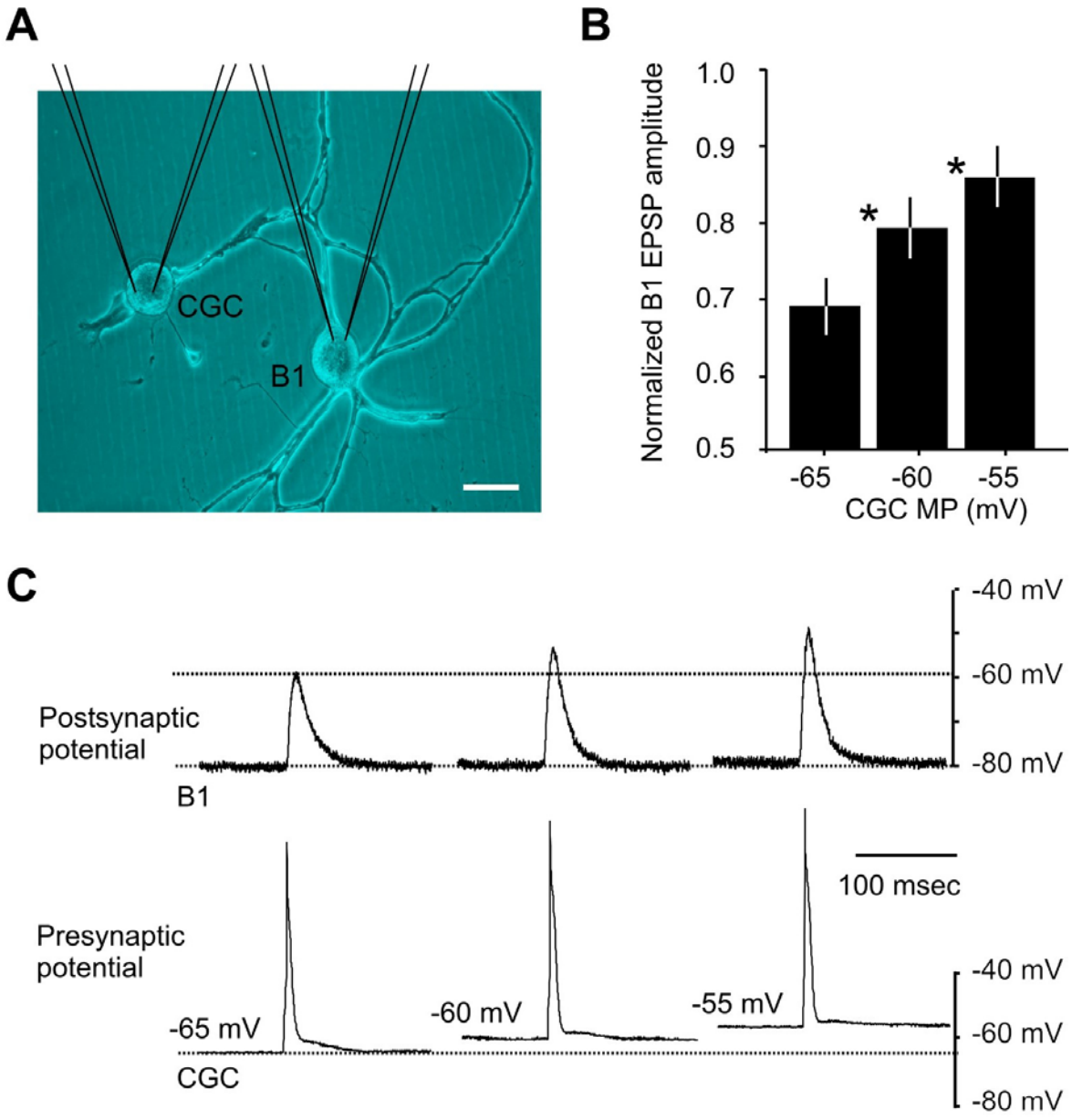

Figure 3. Soma membrane depolarization leads to increased postsynaptic response. A. A pair of 3-day-old co-cultured CGC and B1 feeding motoneurons. Each of the two neurons was impaled with two electrodes to allow both the setting of the membrane potential at predetermined values in both cells and the triggering of single spikes in the CGCs. Scale bar, 100 μM. B. With increasing soma membrane depolarizations, single spikes triggered in the CGC cell body evoke increasingly larger post-synaptic responses in the co-cultured B1 neuron. The CGC membrane potential was increased in 5 mV steps from -65 to -45 mV and B1 EPSP amplitudes, evoked by single triggered CGC spikes, were measured. This graph shows the mean normalized B1 EPSP values (± SEM) at the -65, -60 and -55 mV CGC membrane potential levels. The values at both -60 and -55 mV are significantly (*) larger than at -65 mV (paired t-tests, $p < 0.003$ and 0.01). C. An example of an electrophysiological test showing increasingly larger post-synaptic responses in the B1 neuron to single spikes triggered in the CGC cell body, which was depolarized in 5 mV steps from -65 to -45 mV. Only the traces in the -65 to -55 mV CGC membrane potential range are shown. Note that there is no change in the spike shape with increasing levels of depolarization. (Adapted from figure 6 in Kemenes *et al.* (14), with permission from Elsevier Limited).

Cleary *et al.* (17) showed that the membrane potential of tail motoneurons in *Aplysia* was changed after behavioral long-term sensitization. Behavioral long-term sensitization resulted in a hyperpolarization of the resting membrane potential and a concomitant decrease in spike threshold. These changes would tend to have the opposite effects on motoneuron excitability, so their function in long-term sensitization is so far unclear.

5. CHANGES IN THE THRESHOLD FOR PLATEAU FORMATION

Changes in the threshold for plateau formation in motor pattern generation occurs in the same *Aplysia* interneuron (B51) in both classical and operant conditioning of feeding allowing comparisons to be made of the non-synaptic intrinsic changes occurring in B51 in the two different types of learning (18). Changes in the plateauing properties of B51 are important because the neuron plays a key role in decision making in the *Aplysia* feeding network (18).

During reward classical conditioning in preparations made from behaviorally-trained animals, B51 showed a greater number of plateau potentials compared with controls and it was depolarized more by the CS. Neither the input resistance nor resting potential of the B51 was affected by conditioning but another intrinsic property, the threshold for plateau initiation was increased. This would make the cell less responsive to excitatory synaptic input but nevertheless the cell still showed more plateau potentials after conditioning, so some other unknown factor must overcome this diminished excitability. Similar results were obtained in both *in vivo* and *in vitro* conditioning (19).

Brembs *et al.* (20) developed a behavioral paradigm for reward operant conditioning using the consummatory (ingestive) phase of the feeding cycle as the operant. A cellular correlate of operant conditioning was monitored by intracellularly recording the B51 feeding interneuron in isolated buccal ganglia made from behaviorally-trained animals. Cells from the contingent group show a significant decrease in threshold for plateau formation and a significant increase in input resistance compared with cells from yoked controls. To test whether this is due to an intrinsic change in the B51 cell rather than changes in outside input originating from outside the cell, an analogue of conditioning was developed where B51 was grown in culture and electrically-triggered burst of spikes paired with 6 second puffs of dopamine applied close to the isolated cell over a 10 minute training period. Contingent application of these two stimuli produced a significant reduction in plateau threshold and a significant increase in input resistance compared with unpaired controls similar to that occurring in the previous intact buccal ganglion preparation (20). These results indicate that intrinsic changes are induced in B51 by operant conditioning.

Some information is now available on the molecular mechanisms mediating the dopamine responses in B51 (19). Both the cAMP/PKA and the PKC pathway are involved. Inhibiting PKA with bath application of Rp-cAMP blocks conditioning and conversely injecting cAMP into B51 mimicks conditioning. Blocking PKC with bath application of bisindolymaleimide also blocks conditioning. Because activating the PKA pathway alone can fully mimick the effects of conditioning it appears the PKC is acting up-stream of PKA and it is suggested (19) that the point of convergence is adenylyl cyclase that is known to be coupled to D1 dopamine receptors present on B51.

The two types of conditioning have the opposite types of effect - increasing the plateau membrane potential threshold in classical conditioning and decreasing it in operant conditioning (18) This is despite the increase in overall plateauing frequency in both types of conditioning in the intact network. How these differential effects of the two types of conditioning on B51 plateauing is generated by the feeding network is unknown.

6. CONCLUSIONS AND PERSPECTIVES

We conclude that non-synaptic mechanisms are an important component of molluscan memory. They mainly act to increase the excitability of sensory cells and interneurons 1) by increasing the input resistance of the somal membrane so that synaptic or receptor currents cause greater depolarization and a consequent increase in firing rates, 2) by depolarizing the membrane potential so that the cell is closer to threshold for spike initiation and 3) by reducing the threshold for plateauing so that triggering synaptic inputs increase the occurrence of plateauing potentials. An example where persistent depolarization of membrane potential has no effect on firing rate occurs in the modulatory CGCs of *Lymnaea* after chemical conditioning. Here the persistent depolarization acts to cause presynaptic facilitation of the CS sensory pathway by increasing transmitter release from local CGC proximal neuritic axonal branches rather than by changing CGC firing rate (14).

It is usually assumed that these changes in excitability arise from persistent changes in the strength of intrinsic ionic currents (e.g., B cell photoreceptors of *Hermissenda)*, particularly those mediated by potassium channels. An exception may be the CV1 cells of *Lymnaea* (12). The input resistance of these cells is unaffected, despite a persistent depolarization following conditioning. This may indicate that the intrinsic ion channel properties of CV1 are not involved although there are a number of examples where long-lasting depolarization occurs with no net increases in input resistance (e.g., 21, 22). The persistent change in CV1 membrane potential could have its origins in enhanced extrinsic synaptic input although this seems unlikely as synaptic input would be expected to change input resistance.

How can changes in general electrical excitability lead to a defined conditioned response to a specific CS? This is not a problem when the excitability change occurs in sensory neurons that form the CS pathway, such as the B type photoreceptors that mediate the photo-tactic conditioned response in *Hermissenda* (3) or the touch sensitive neurons involved in classical conditioning of the siphon-gill withdrawal reflex of *Aplysia* (9). When excitability changes occur in command-like neurons like CV1s (12), a change in excitability might generalize to a wider range of sensory inputs other than those used for the original conditioning experiments. In the example of the CV1 cells of the *Lymnaea* feeding system, the selectivity of

the CS response to lip touch after conditioning is ensured by CV1 being embedded in a small network with a highly specialized function that responds to only a narrow range of food-related sensory inputs. These sensory inputs arise mainly from the lips, that normally come into contact with food during rhythmic feeding, unlike other sensory structures like the tentacles. Indeed, in behavioral conditioning experiments (23), no generalization of the classically conditioned response occurred from lips to tentacles. In further electrophysiological experiments, artificial depolarization of the CV1 cells in naive snails selectively increases the response to lip touch with no effect on tentacle touch (12). This specificity of the conditioned response appears to be due to the selective ability to reinforce synaptic inputs form one site on the body versus another even within the same sensory modality.

Another question that arises is whether changes in non-synaptic properties of neurons in molluscs are important for specific phases of memory formation as has been suggested in vertebrate systems (24). Evidence for this comes from recent work on the CGCs in the *Lymnaea* feeding system (14). The use of a single trial training protocol allowed us to follow both the onset and persistence of neuronal changes that paralleled the time course of long-term memory formation measured behaviorally. The delayed onset of the depolarization of the CGC shows that it is not involved in the early expression of the memory trace or any consolidation process, suggesting that the CGCs are involved in long-term memory alone. While long-term memory may be supported by the depolarized state of the CGCs, early memory and memory consolidation must involve other mechanisms. This could involve synaptic plasticity as has been demonstrated in *Lymnaea* aversive conditioning (25). If these synaptic changes also persist in parallel with long-term memory, the role of the learning-induced membrane potential depolarization of the CGCs may add a type of extrinsic non-synaptic plasticity to the feeding circuit to support or provide a back-up for learning-induced synaptic plasticity.

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