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Source: Zoological Science, 34(1):72-80.
Published By: Zoological Society of Japan
DOI: http://dx.doi.org/10.2108/zs160152
URL: http://www.bioone.org/doi/full/10.2108/zs160152

ZOOLOGICAL SCIENCE **34**: 72–80 (2017)

Cerebral Giant Cells Are Necessary for the Formation and Recall of Memory of Conditioned Taste Aversion in *Lymnaea*

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The pond snail *Lymnaea stagnalis* can acquire conditioned taste aversion (CTA) as a long-term memory. CTA is caused by the temporal pairing of a stimulus, such as sucrose (the conditioned stimulus; CS), with another stimulus, such as electric shock (the unconditioned stimulus; US). Previous studies have demonstrated changes in both cellular and molecular properties in a pair of neurons known as the cerebral giant cells (CGCs), suggesting that these neurons play a key role in CTA. Here we examined the necessity of the pair of CGC somata for the learning, memory formation and memory recall of CTA by using the soma ablation technique. There was no difference in the feeding response elicited by the CS before and after ablation of the CGC somata. Ablation of the CGC somata before taste-aversion training resulted in the learning acquisition, but the memory formation was not observed 24 h later. We next asked whether memory was present when the CGC somata were ablated 24 h after taste-aversion training. The memory was present before performing the somata ablation. However, when we tested snails five days after somata ablation, the memory recall was not present. Together the data show that: 1) the somata of the CGCs are not necessary for learning acquisition; 2) the somata are necessary for memory formation; and 3) the somata are necessary for memory recall. That is, these results demonstrate that the CGCs function in the long-term memory of CTA in *Lymnaea*.

Key words: cerebral giant cell, conditioned taste aversion, long-term memory, *Lymnaea*, soma ablation

INTRODUCTION

The pond snail *Lymnaea stagnalis* is an excellent animal to use as a model system for studying causal cellular and molecular mechanisms in learning and memory (Kojima et al., 1998; Benjamin et al., 2008; Sunada et al., 2010a; Lukowiak et al., 2014; Yamagishi et al., 2015). *Lymnaea* learn and form long-term memory (LTM) following aversive classical conditioning in which they are taught not to respond to a food substance that normally elicits a feeding response. This behavior is referred to as conditioned taste aversion (CTA) (Yamanaka et al., 1999; Sugai et al., 2007; Ito et al., 2015b; Kojima et al., 2015). To produce CTA in snails, an appetitive stimulus (e.g., sucrose) is used as the conditioned stimulus (CS). Application of the CS to the lips increases the feeding response (i.e., the number of bites) (Kojima et al., 1996). An aversive stimulus (e.g., electric shock) is used as the unconditioned stimulus (US). The US causes the snails to immediately stop feeding (Takigami et al., 2013). In the taste-aversion training procedure, the CS is paired with the US. After repeated CS-US pairings, the CS no longer elicits feeding, and the memory (i.e., not to respond to the CS) persists for at least a month (Ito et al., 1999).

In a recent review article (Kojima et al., 2015), we have described in some detail the neural mechanisms underlying CTA: the cerebral giant cells (CGCs) have been hypothesized to play some key roles in the initial learning process and the subsequent memory formation and recall (Kojima et al., 2001). For example, induced activation of the CGCs brought about a larger and longer lasting polysynaptic inhibitory postsynaptic potential (IPSP) in the neuron 1 medial (N1M) cell via the neuron 3 tonic (N3t) cell in taste aversion-trained snails compared to the IPSP recorded in control snails (Kojima et al., 1997; Ito et al., 2013). The N1M cell and the N3t cell are members of the feeding central pattern generator (CPG) and act in the N1 (protraction) and N3 (swallow) phases, respectively (Yeoman et al., 1996). These data were consistent with the hypothesis that an enhanced IPSP in the N1M cell suppressed the feeding behavior in CTA (Ito et al., 2012). Further, the release of serotonin from the CGCs to their follower neurons was found to be controlled by levels of cAMP, protein kinase A (PKA), cAMP

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doi:10.2108/zs160152

ABBREVIATIONS

C/EBP, CCAAT enhancer binding protein; CGC, cerebral giant cell; CNS, central nervous system; CPG, central pattern generator; CS, conditioned stimulus; CTA, conditioned taste aversion; CREB, cAMP response element binding protein; IPSP, inhibitory postsynaptic potential; LTM, long-term memory; N1M, neuron 1 medial; N3t, neuron 3 tonic; PKA, protein kinase A; PeD12, pedal-dorsal 12 interneuron; PlB, pleuro-buccal interneuron; RPeD1, right pedal-dorsal 1 interneuron; RPeD11, right pedal-dorsal 11 interneuron; US, unconditioned stimulus.

response element binding protein (CREB), and probably an involvement of the CCAAT enhancer binding protein (C/EBP), consistent with the hypothesis that these signaling cascades play key molecular roles in mediating CTA (Nakamura et al., 1996a; Hatakeyama et al., 2004, 2006; Sadamoto et al., 2004, 2010; Azami et al., 2006; Otsuka et al., 2013).

In addition to CTA, *Lymnaea* are also capable of appetitive classical conditioning of feeding behavior (Benjamin et al., 2000) and operant conditioning of aerial respiration (Lukowiak et al., 1996). In the operant conditioning procedure, snails learn to decrease their aerial respiratory activity as they receive a tactile stimulus to the pneumostome area (i.e., the breathing tube) each time they attempt to open it to perform aerial respiration (Lukowiak et al., 2003, 2006). This negative reinforcement causes the snails to close the pneumostome. The number of attempted pneumostome openings is counted in the training and memory retention sessions. Memory is operationally defined as a significant decrease in the number of attempted openings in the memory test sessions compared to the initial training session. *Lymnaea* are capable of this associative learning and consolidating the learning into LTM (Sangha et al., 2003a).

The 3-neuron circuit in the aerial respiratory CPG has been experimentally shown to be both necessary and sufficient (Syed et al., 1990, 1992). Moreover, the neuron that initiates the rhythmic activity in this circuit, right pedal-dorsal 1 interneuron (RPeD1), has been shown to have significantly altered activity consistent with the decreased respiratory behavior as a result of operant conditioning (Spencer et al., 1999, 2002). Further, it has been demonstrated that this neuron is a necessary site for LTM formation of aerial respiratory behavior (Scheibenstock et al., 2002). In that study, the authors employed a surgical technique in otherwise intact snails to ablate the soma of RPeD1, leaving behind a functional neurite. These snails continued to be able to perform aerial respiratory activity and they could learn and form a 3 h memory, but they could not form LTM. The 3 h memory has been termed intermediate term memory (ITM) and has been shown to be dependent on new protein synthesis; whereas LTM was found to be dependent on both altered gene activity and new protein synthesis (Sangha et al., 2003a). Thus, with soma ablation the remaining functional neurites have the ability to translate mRNA into new proteins (Van Minnen et al., 1997). However, with soma ablation the nucleus is no longer present and there cannot be altered gene activity, thus LTM cannot occur. It was with this strategy that the Lukowiak group showed that RPeD1 was a necessary site for reconsolidation, extinction, and forgetting (Sangha et al., 2003b, c, 2005).

In the present study, we employed a soma ablation strategy to determine if the CGCs were necessary for the learning, memory formation and memory recall of CTA. Our data are consistent with the hypotheses that the CGC somata are not necessary for learning, but are necessary for both the formation and recall of CTA memory.

MATERIALS AND METHODS

Taste-aversion training procedure

Specimens of *Lymnaea stagnalis* (Linnaeus, 1758) with an 18–23 mm shell obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam) were used in the present study (Sugai et al., 2007; Ito et al., 2012). All snails were maintained in dechlorinated tap water (i.e., pond water) under a 12:12 light-dark cycle at 20°C and fed *ad libitum* on turnip leaves (*Brassica rapa* var. *peruviridis*; Komatsuna [in Japanese]) and a commercially available product called Spiral Shell Food (a combination of seaweed, brewer's yeast and vitamins; Nisso, Saitama, Japan) every other day. *Lymnaea* exhibit good growth and reproduction under these conditions.

Recently, an automated learning apparatus for the taste-aversion training procedures has been established by the Sakakibara group (Takigami et al., 2016). The same apparatus was used in the present study, with a slight modification. An application of sucrose (100 mM; CS) for 5 s and an application of electrical shock (9 V, 0.4 μA; US) for 0.2 s were paired (Takigami et al., 2013). The inter-stimulus interval was 5 s, and the inter-trial interval was 65.2 s. We starved snails for one day before training and then paired the CS with the US (Mita et al., 2014a, b). The snails received 10 paired presentations of the CS-US (Wagatsuma et al., 2004). To assess the learning and the memory retention of CTA, the feeding response (i.e., the number of bites) over 1 min after application of the CS was counted at each of 10 min, 24 h, and five days following the training. Previous control experiments (i.e., a backward training (US-CS) and naive training in which snails receive only distilled water as both the CS and US) have shown that only when the sucrose and electrical shock (i.e., CS-US) are paired in a forward manner does the learning and memory formation occur (Ito et al., 2015a).

CGC somata-ablation procedure

The somata ablation procedure was performed in almost the same manner described in the previous paper by the Lukowiak group (Scheibenstock et al., 2002), with a slight modification. Snails were anesthetized in ice for 20 min with 1–1.5 ml of 50 mM $MgCl_2$ that was injected through the foot. This anesthetization allowed a dorsal midline incision to expose the snail's brain. We ablated a pair of CGC somata by gently poking with a hand-held glass microelectrode. The incision was small enough to allow spontaneous recovery without suturing. Snails began to wake from the effects of the anesthetic within several hours of the surgery. The recovery period after the surgery was five days, because the snails that underwent an operation exhibited good feeding and reproduction 5 days later. Then we carried out the taste-aversion training and the memory test.

Data analysis

Data are expressed as the mean ± SEM. The significant difference was set at $P < 0.05$. A paired *t*-test was used to compare the feeding responses to the CS between the non-ablated snails and the snails at 5 days after the CGC somata ablation. For the results of taste-aversion training, the Bartlett test, the Friedman test and the post hoc Tukey's multiple comparison test were performed (GraphPad Prism ver. 5, GraphPad Software, San Diego, CA, USA; JSTAT for Windows ver. 10.0, http://toukeijstat.web.fc2.com/index.html). Because the Bartlett test offered us the inhomogeneity for variance of the data to be compared ($P < 0.05$), we used the Friedman test for the results of taste-aversion training. On the other hand, for the comparison of pre-tests, the Bartlett test, one-way ANOVA and the post hoc Scheffé test were performed. In this case, the Bartlett test ensured the homogeneity for variance of the data ($P > 0.05$), and thus we used one-way ANOVA.

RESULTS

Effects of CGC somata ablation on the feeding response to sucrose solution

We ablated the pair of CGC somata in intact snails following the procedure described in the Methods (Fig. 1A).

The sham (i.e., control) operation in which only the skin was cut demonstrated that snails recovered completely from the incision in five days, and that the pair of CGC somata were still present (arrowheads in Fig. 1B). To ablate the CGCs following the incision, a hook was applied to the cerebral commissure to pull out the central nervous system (CNS). We then used a hand-help micropipette to probe the soma of each CGC. This resulted in ablation of the soma. As can be seen in Fig. 1C, 5 days after the GCG somata were ablated their presence could not be detected. The snails showed recovery in five days following the somata ablation procedure, because they exhibited good feeding and reproduction five days later.

We next investigated whether the CGC somata-ablated snails were able to respond to a sucrose solution. These data are presented in Fig. 2. As can be seen, the feeding response in snails (n = 11) elicited by the sucrose solution was statistically the same before and 5 days after CGC somata ablation. These data are consistent with the hypothesis that the feeding response elicited by the sucrose CS is not dependent on the presence of the CGC somata.

Effects of CGC somata ablation before training on the learning acquisition and memory formation of CTA

We used three separate groups of snails: 1) a no-surgery control, 2) a sham-operated control, and 3) a CGC somata-ablated group. In all groups, five days after the surgery we counted the feeding response for 1 min following the application of the CS (i.e., 100 mM sucrose for 5 s). This was plotted as the pre-test. Ten minutes later, we initiated taste-aversion training in the three groups. We tested the response to the CS at 10 min and at 24 h after the end of the training procedure. The time line for these experiments is shown in Fig. 3A.

Figure 3B shows the results of taste-aversion training in the three groups. The response (i.e., number of bites) in each group (Fig. 3B(a–c)) elicited by the CS in the pre-test session was statistically similar across all the groups (one-way ANOVA: $F(2,31) = 3.42$, $P > 0.05$). The response in each group in the 10 min post-test following taste-aversion training showed a significant decrease compared to the number of bites in the pre-test (for the no-surgery group, Friedman test: $\chi^2 = 10.7$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, n = 11; for the sham-operated group, Friedman test: $\chi^2 = 16.1$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, n = 12; for the CGC somata-ablated group, Friedman test: $\chi^2 = 14.9$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, n = 12). That is, in each group there was a significant decrease in the feeding response elicited by the CS 10 min after taste-aversion training (Fig 3B). This indicates that all three groups had the ability to learn CTA. These data are consistent with the hypothesis that the CGC somata are not necessary for taste-aversion learning (Fig. 3B(c)).

However, when we tested whether memory formed in the three groups, we found that one group, the somata-ablated group, did not form memory. When we tested the feeding response to the CS 24 h after training, we

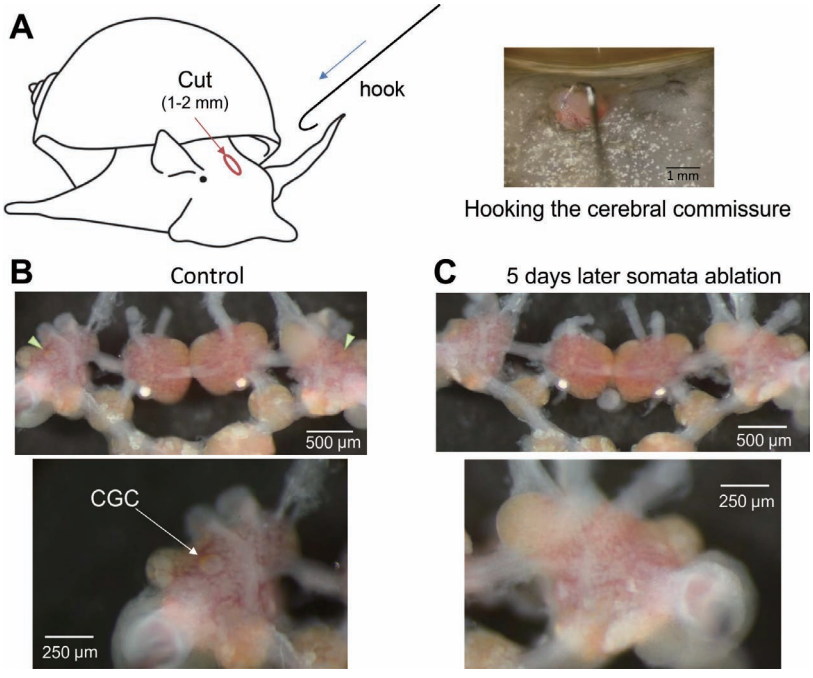

Fig. 1. Schematic presentation of the soma ablation surgery. **(A)** The CNS was pulled outside of the body by hooking the cerebral commissure. The skin of head was cut about 1–2 mm; the hook was inserted into the cut; and it hooked up the cerebral commissure. We could easily find a pair of CGCs in the cerebral ganglia and ablated them by poking. **(B)** In the control group, the CGCs were observed both in the left and right cerebral ganglia (arrowheads). A pair of silver balls are statocysts located in the pedal ganglia. A pair of pleural ganglia, a pair of parietal ganglia and a visceral ganglion, but not a pair of buccal ganglia, are observed in the photo. In a magnified view of the left cerebral ganglion, the arrow indicates the CGC cell body, or soma. The CGC is easy to detect from the size of the cell body. **(C)** The upper photo shows the exteriorly relocated CNS of a snail at five days after the CGC somata-ablation surgery. The lower photo shows the absence of spontaneous recovery of the cell bodies in the somata-ablated group.

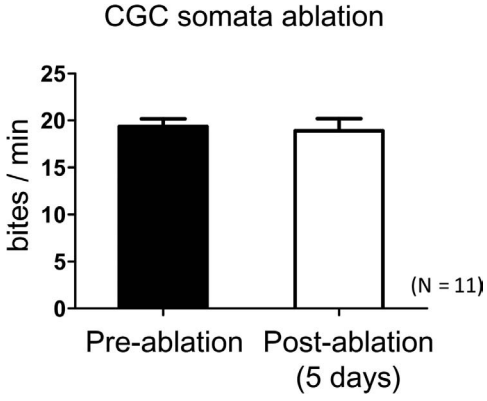

Fig. 2. CGC somata ablation does not alter feeding behavior. The feeding responses to the CS were counted before and five days after ablation of the somata of CGCs. No changes ($P > 0.05$) in the parameters of feeding behavior were seen following the surgical procedure and the somata ablation.

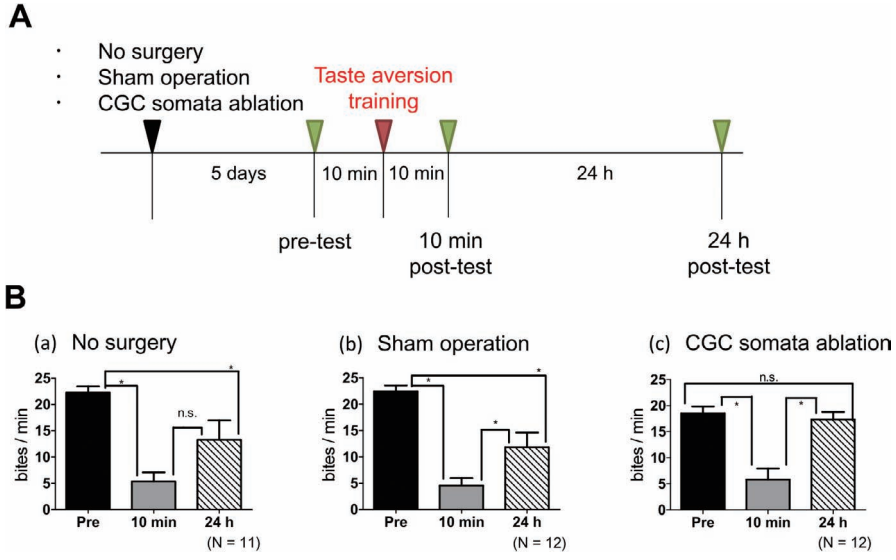

Fig. 3. CGC somata-ablated snails cannot form memory 24 h after taste-aversion training. **(A)** The time chart of the CGCs somata ablation or sham operation, pre-test, 10 min post-test and 24 h post-test is shown. The snails were first tested (i.e., pre-test) five days after the surgery. The 10 min post-test was performed 10 min after the taste-aversion training. The 24 h post-test was performed 24 h after the taste-aversion training. **(B)** The number of feeding responses to the CS: (a) no-surgery group, (b) sham-operated group, (c) CGC somata-ablated group. *$P < 0.05$.

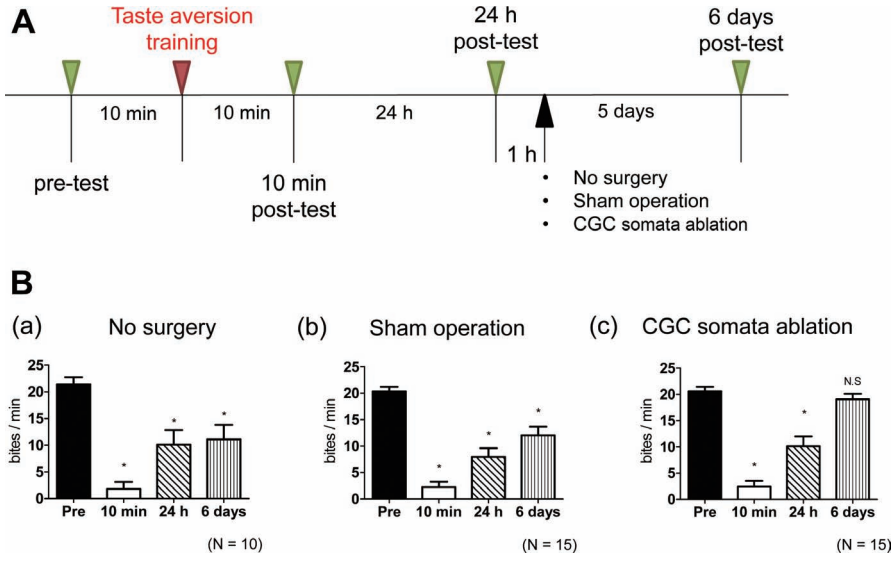

Fig. 4. CGC somata-ablated snails cannot recall memory five days after surgery. **(A)** The time chart of the CGC somata ablation or sham operation. Both somata ablation and sham operation surgery were carried out after the 24 h post-test. The number of bites elicited by the CS was counted five days after surgery (i.e., six days post-test). **(B)** The number of feeding responses to the CS: (a) no-surgery group, (b) sham-operated group, (c) CGC somata-ablated group. *$P < 0.05$, compared with the pre-test value.

found that no-surgery group and sham-operated group showed a significant decrease in the feeding behavior elicited by the CS in comparison with the pre-test (Tukey's multiple comparison test: $P < 0.05$; Fig. 3B(a), (b)). As can be seen, the scores at 24 h in these control groups were slightly increased from those at 10 min (Fig. 3B(a), (b)), but the CTA was retained at least until 24 h after the training. Therefore, the results showed that memory was formed in both control groups. However, in the CGC somata-ablated group, we found that memory was not present. That is, the number of bites elicited by the CS in the 24 h post-test was not statistically different from that in the pre-test (Tukey's multiple comparison test: $P > 0.05$) and was significantly greater than the response in the 10 min post-test (Tukey's multiple comparison test: $P < 0.05$, $n = 12$). These data are consistent with the hypothesis that the CGC somata-ablated snails could not consolidate the associative learning into memory, and thus CGC somata are necessary for the memory formation of CTA.

Effects of CGC somata ablation after training on the memory recall of CTA

As described above, the data support the hypothesis that CGC somata are necessary for memory formation following the taste-aversion training. Next, we asked whether the CGC somata are necessary for memory recall. That is, if a memory forms and then the CGC somata are then ablated, will snails continue to have the ability to recall the formed memory? We again employed three groups of snails: 1) no-surgery, 2) sham-operated, and 3) CGC somata-ablated snails, but in all three groups the surgeries were performed on the snails after memory formed (Fig. 4A).

One hour after the 24 h post-test, the pair of CGC somata were ablated in the CGC somata-ablated group, whereas in the other two groups the surgeries were also performed 1 h after the 24 h post-test session. We tested for LTM 5 days later in order to allow recovery from the various surgeries (i.e., the 6 days post-test after the taste-aversion training). The response in each group (Fig. 4B(a–c)) elicited by the CS in the pre-test sessions was statistically similar across all the groups (one-way ANOVA: $F(2,37) = 0.29$, $P > 0.05$). As can be seen in Fig. 4B(a), (b), LTM was present at the six days post-test session in both the no-surgery group and the sham-operated group. That is, in the no-surgery group (Friedman test: $\chi^2 = 17.7$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, $n = 10$), the number of bites elicited by the CS in all three post-tests (10 min, 24 h and 6 days) was significantly less

than in the pre-test session. Similarly, in the sham-operated group (Friedman test: $\chi^2 = 31.0$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, $n = 15$), the number of bites elicited by the CS in all three post-tests (10 min, 24 h and six days) was significantly less than in the pre-test session.

However, in the CGC somata-ablated group, the number of bites elicited was different from that in the pre-test session only in the 10 min and 24 h post-test sessions (Friedman test: $\chi^2 = 34.6$, $P < 0.01$, Tukey's multiple comparison test: $P < 0.05$, $n = 15$). That is, in the CGC somata-ablated group, the number of bites elicited by the CS in the six days post-test session was not significantly different from the number elicited in the pre-test session (Fig. 4B(c)). Furthermore, in the CGC somata-ablated group, there was a significant difference in the number of bites elicited between the 24 h post-test and the six days post-test session (Tukey's multiple comparison test: $P < 0.05$). This was in contrast to the findings in the two control groups. In those two groups, there were no differences between the 24 h post-test and 6 days post-test (Tukey's multiple comparison test: $P > 0.05$). These data are not consistent with the hypothesis that the CGCs are unnecessary for LTM recall. In Fig. 4A, B(c), we should note that there was a 1 h interval after the memory test (i.e., 24 h post-test) before the paired CGC somata were ablated. Previously, experiments by the Lukowiak group have shown that a 1 h interval is a sufficient time period not to interfere with consolidation, reconsolidation or attaining a 'false' memory (Scheibenstock et al., 2002; Sangha et al., 2003b, 2005; Lukowiak et al., 2007). Thus, our results are unlikely to be due to aversive side-effects of the ablation procedure.

DISCUSSION

A previous study showed that new protein synthesis and altered gene activity in the CGCs were important for CTA-LTM in *Lymnaea* (Azami et al., 2006; Lukowiak et al., 2007). Here we have built on this previous finding by showing that the CGC somata are not necessary for taste-aversion learning to occur, but are necessary both for memory formation following learning and for memory recall following memory formation. These findings allowed us to construct a new cellular model for CTA learning and memory in *Lymnaea* (Fig. 5). Although we strongly believe that CGC somata are necessary for memory formation following the taste-aversion training, there is room for further study, with a small possibility, if the CGC neurites die between 5 and 6 days after the CGC somata ablation (Fig. 3).

The majority of synaptic interactions in molluscs occur on the neurites, and thus the soma of a particular neuron can be removed without altering synaptic interactions (Scheibenstock et al., 2002; Bullock and Horridge, 1965). Therefore, in the classical conditioning procedure (i.e., taste-aversion training), in which pairings of the CS with US in close temporal contiguity lead to the CS pre-

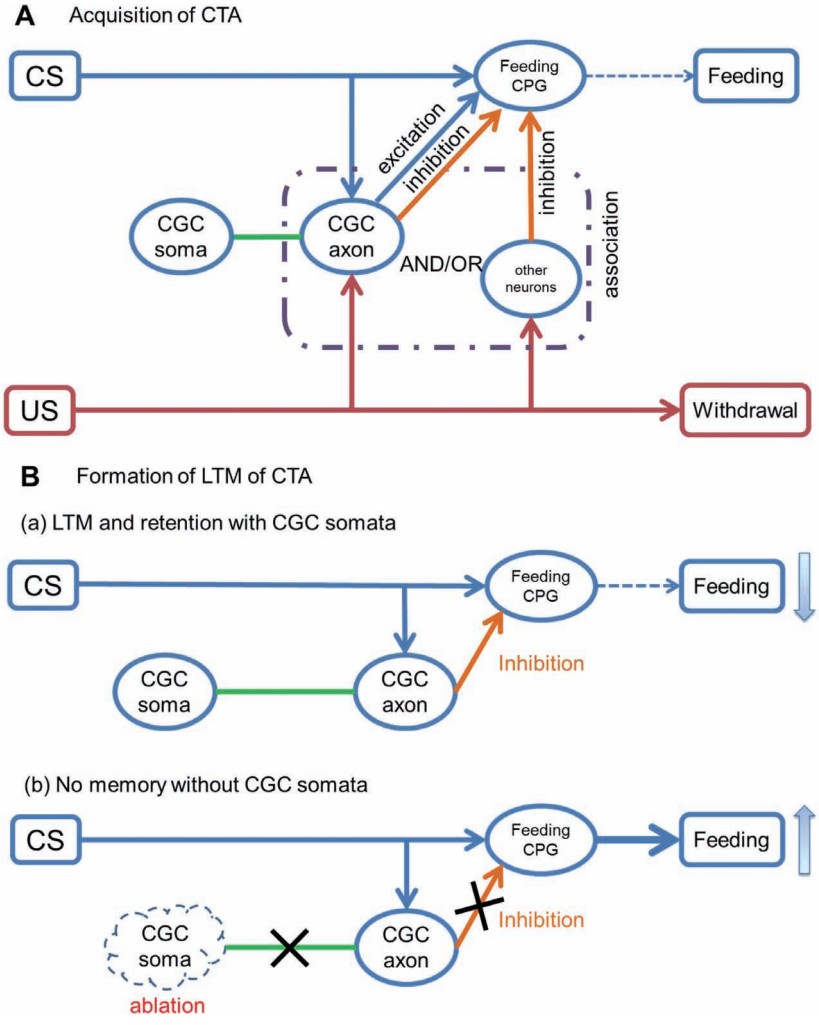

Fig. 5. Schematic diagrams of the role of the CGC somata and axon (neurite) in the learning acquisition, memory formation and memory recall of CTA. **(A)** The role of the CGC somata and the axon (neurite) in learning acquisition. The information of the CS (blue arrow) and the US (red arrow) were associated in the CGC axon (neurite) and/or other neurons. At the time point of learning acquisition, inhibitory inputs from the CGC axon (neurite) and/or other neurons to the feeding CPG (orange arrow) lead to a decrease in feeding behavior (blue dotted arrow). This behavioral reduction occurred regardless of the presence or absence of CGC somata. The green line between the CGC soma and the CGC axon (neurite) represents a mutual interaction. Further, we should note that the 'monosynaptic' input from the CGC to the N1M cell, which is one of the feeding CPG neurons, is excitatory, but the 'polysynaptic' input is inhibitory. This polysynaptic inhibition has been shown to be larger and to last longer in conditioned snails than in control snails (Kojima et al., 1997). **(B)** The role of the CGC somata in memory formation and recall. (a) The CGC somata functioned in the inhibitory input pathway from their axons (neurites) to the feeding CPG, resulting in the reduction of the feeding response. LTM formation requires both altered gene activity, which of course depends on having a nucleus, and new protein synthesis. (b) In contrast, the CGC somata ablation caused the loss of the long-lasting inhibitory input from the CGC axon (neurite) to the feeding CPG. Snails did not form or recall memory when the CGC somata were lost.

dicting the onset of the US, associative learning occurs as a result of synaptic interactions occurring on the neurites of the CGCs. Thus, removal of the somata of the CGCs will not alter the ability of the CGCs to elicit the feeding response, nor alter the ability of the US to cause cessation of feeding (Figs. 2 and 5A). Moreover, the synaptic interactions that lead to associative learning can continue to occur, as they only involve the surviving neurites. We know that learning does not require altered gene activity and new protein synthesis (Milner et al., 1998), and therefore learning would not be compromised in the absence of the CGC somata. It has previously been shown in an aerial respiratory conditioning of *Lymnaea* that memory persisting for up to 3 h requires only new protein synthesis (Sangha et al., 2003a), and we know that the isolated neurites of *Lymnaea* neurons are not only capable of de novo protein synthesis (Van Minnen et al., 1997) but can survive for up to at least 10 days (Scheibenstock et al., 2002). Thus, it is not surprising that short-lasting memory can be elicited in snails with ablated CGC somata.

We conclude that one of these association sites is the CGC neurite for three reasons. (a) As can be seen in Figs. 3 and 4, the CGC somata are necessary for the formation and recall of memory. This is perhaps the most compelling evidence that the CGC neurite is an association site. The association information may retrogradely travel to the somata as described below. (b) An intracellular recording using the CGC soma previously showed that the CS and the US were associated (Nakamura et al., 1999a). Further, the inhibitory pathway from the right pedal-dorsal 11 interneuron (RPeD11) to the CGC has been clarified (Sunada et al., 2012). The RPeD11 is involved in the withdrawal response. (c) Even after the ablation of the CGC somata, the neurites of CGCs are thought to remain alive. Molluscs have long neurites, and in one report, these neurites were demonstrated to function 10 days after soma ablation of the RPeD1 in *Lymnaea* (Scheibenstock et al., 2002).

LTM formation, on the other hand, requires both altered gene activity and new protein synthesis (Fig. 5B(a)) (Sangha et al., 2003a; Costa-Mattioli and Sonenberg, 2008). Thus, because the somata were removed and therefore the nuclei housing the genes were also gone (Fig. 5B(b)), it is not surprising that LTM could no longer be formed. This finding strongly suggests that the CGCs play key roles in the learning, memory formation and memory recall of CTA, and reinforces previous findings showing that the CGCs play key roles in CTA (Ito et al., 2013). Previously, Nakamura et al. showed using intracellular recordings from the CGCs that both the CS and US were associated in these neurons (Nakamura et al., 1999a). It was more recently shown (Sunada et al., 2012) that activity evoked in the RPeD11, a neuron in the network that controls the whole-snail withdrawal response (Inoue et al., 1996; Sunada et al., 2010b), causes a synaptic response in the CGCs consistent with shutting down feeding behavior. It is thought that the interactions of the synaptic inputs triggered by the CS and US bring about molecular changes in the neurites that are then retrogradely transported to the soma, where they can cause alterations in gene activity that in turn are the molecular causes of LTM formation (Kandel et al., 2001). At present we cannot say with any certainty that LTM formation following CTA is solely due to changes occurring with the CGCs, only that the CGCs are a necessary site for LTM formation. We do know that other as-yet-unidentified neurons are involved in taste discrimination for CTA (Sugai et al., 2006).

A series of electrophysiological studies by the Benjamin group have also shown that the CGCs play important roles in both mediating feeding behavior and LTM formation of appetitive food conditioning. For example, using a fine wire attached to the CGC soma to record its activity in a freely behaving snail along with serotonergic antagonists, they demonstrated that the CGC and its neurotransmitter serotonin have a modulatory function in the feeding system of *Lymnaea* (Yeoman et al., 1994a). In addition, when they used a photo-inactivation technique to kill the CGCs (i.e., both the soma and neurites) with a blue laser, the fictive feeding rates 30 min after photo-inactivation were significantly lower than those in controls (Yeoman et al., 1994b). These data are consistent with our hypothesis that the CGCs play a modulatory role in controlling the frequency of the CPG that drives feeding behaviors. Finally, using a single-trail training procedure the Benjamin group concluded that both synaptic and non-synaptic connections (e.g., changes in intrinsic membrane properties) of the CGCs were crucial for mediating behavior.

The critical findings with respect to the CGC under the appetitive feeding conditioning were as follows. A single-trial classical appetitive conditioning leads to a delayed somal membrane potential depolarization in the CGC (Kemenes et al., 2006). The use of a single-trial protocol enabled the researchers to follow both the onset and persistence of neuronal changes that paralleled the time course of LTM. The depolarization of the CGC emerged between 20 and 24 h after training, and it was sufficient to lead to a prolonged intracellular Ca^{2+} increase in the proximal CGC neurite terminal, which in turn was involved in the enhanced response to the CS that occurred after learning. Thus, LTM was thought to be supported by this delayed non-synaptic plasticity in the reward feeding conditioning. Taken together, these data suggest that CGCs are crucial for adaptive changes in feeding behaviors (i.e., CTA and appetitive classical conditioning) in *Lymnaea*.

We previously found that molluscan insulin-related peptides (MIPs) were up-regulated in snails exhibiting CTA (Azami et al., 2006). We next found that application of mammalian insulin or MIPs harvested from *Lymnaea* to the isolated CNS caused long-term synaptic enhancement at the serotonergic excitatory monosynaptic connection between the CGCs and the buccal 1 (B1) motor neuron (Murakami et al., 2013a). That is, the excitatory postsynaptic potentials (EPSPs) recorded in the B1 motor neuron elicited by activation of the CGCs were significantly increased when insulin or MIPs were applied to the CNS. This change in synaptic efficacy was hypothesized to be the neural correlate of behavioral CTA and is thought to underlie the CTA-LTM consolidation process (Murakami et al., 2013a; Kojima et al., 2015). Further, the paired pulse ratio was unaltered following insulin application, suggesting that the effects of insulin on synaptic plasticity are mediated 'post-synaptically' in the B1 motor neuron (Hatakeyama et al., 2013; Murakami et al., 2013b). Thus, the transcription and translation mechanisms in CGC follower neurons located in the buccal gan-

glia will be the future targets of CTA studies.

Recently, other two interneurons in addition to the CGCs were implicated in suppressing the feeding CPG in *Lymnaea*. The first one is an interneuron termed the pleurobuccal interneuron (PlB), which has extensive inhibitory synaptic connections with interneurons and motor neurons of the feeding network (Alania et al., 2004a, b). The second interneuron has been identified as the pedal-dorsal 12 interneuron (PeD12). This interneuron has been shown to play a critical role in behavioral choice. That is, its activity underlies the competitive interactions between the otherwise autonomous feeding and withdrawal-response networks (Pirger et al., 2014). In particular, the PeD12 activates the PlB upon strong aversive stimulation, suggesting a cellular/network mechanism for the suppression of feeding following an aversive stimulus such as the electric shock to the snail used our CTA paradigm. In the future we plan to examine the involvement of both the PlB and the PeD12 interneurons in the cellular mechanisms underlying CTA.

Finally, we emphasize that the neurons (i.e., the CGCs) whose somata were ablated have been shown to be serotonergic (Hatakeyama et al., 1999; Kawai et al., 2011). Thus, the ablation of the somata of these two neurons results in a partial loss of the serotonin supply. Because CTA in *Lymnaea* has been hypothesized to involve in a change of serotonin release onto their follower neurons (Kojima et al., 1997), the data we have obtained with removal of the CGC somata are consistent with our earlier findings. Further, serotonin release from the CGCs is altered by changes in PKA and CREB activities (Nakamura et al., 1999b; Sadamoto et al., 2004), and these findings are consistent with our findings using an RNAi technique (Wagatsuma et al., 2006). Because the removal of the CGC somata removes thus the nuclei and thus the genes, altered gene activity cannot occur; as well serotonin release may be attenuated.

In conclusion, the data presented here are consistent with our long-held hypothesis that the CGCs are required for CTA-LTM. Our data further suggest that the sites where the association occurs between the CS and the US in taste-aversion training is most likely the CGC neurites. In the future, we will attempt to determine the causal molecular changes occurring in the CGC somata that underlie CTA learning and memory formation in *Lymnaea*.

ACKNOWLEDGMENTS

This work was supported by KAKENHI grants from the Japanese Society for the Promotion of Science (24657055 and 25291074) to E.I., Waseda University grants for Specific Research Projects [2016B-068, 2016B-069 and 2016S-037 to E.I.], and a grant from the Natural Sciences and Engineering Research Council of Canada (227993-2013) to K.L.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

HS, KL and EI conceived and designed the experiments. HS performed the experiments. HS and EI analyzed the data. HS, KL and EI wrote the paper.

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(Received September 13, 2016 / Accepted October 18, 2016)