

Taste discrimination in conditioned taste aversion of the pond snail *Lymnaea stagnalis*

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Accepted 3 January 2006

Summary

Conditioned taste aversion (CTA) in the pond snail *Lymnaea stagnalis* has been widely used as a model for gaining an understanding of the molecular and behavioral mechanisms underlying learning and memory. At the behavioral level, however, it is still unclear how taste discrimination and CTA interact. We thus examined how CTA to one taste affected the feeding response induced by another appetitive food stimulus. We first demonstrated that snails have the capacity to recognize sucrose and carrot juice as distinct appetitive stimuli. We then found that snails can become conditioned (i.e. CTA) to avoid one of the stimuli and not the other. These results show that snails can distinguish between appetitive stimuli during CTA, suggesting that taste discrimination is processed

upstream of the site where memory consolidation in the snail brain occurs. Moreover, we examined second-order conditioning with two appetitive stimuli and one aversive stimulus. Snails acquired second-order conditioning and were still able to distinguish between the different stimuli. Finally, we repeatedly presented the conditional stimulus alone to the conditioned snails, but this procedure did not extinguish the long-term memory of CTA in the snails. Taken together, our data suggest that CTA causes specific, irreversible and rigid changes from appetitive stimuli to aversive ones in the conditioning procedure.

Key words: conditioned taste aversion, long-term memory, *Lymnaea stagnalis*, second-order conditioning, taste discrimination.

Introduction

The pond snail *Lymnaea stagnalis* is capable of acquiring different forms of associative learning, including both classical and operant conditioning. In addition to acquisition of new behavior (i.e. learning) they are able to consolidate the learning into long-term memory (i.e. the ability to recall the new behavior) (Benjamin et al., 2000; Jones et al., 2003; Lukowiak et al., 2003; Fulton et al., 2005; McComb et al., 2005a; McComb et al., 2005b; Parvez et al., 2005; Sakakibara et al., 2005). Our team has studied another form of classical conditioning and its consolidation into long-term memory, namely conditioned taste aversion (CTA) (Ito et al., 1999). To produce CTA in *Lymnaea*, an appetitive stimulus (e.g. sucrose) is used as the conditional stimulus (CS). Application of CS to the lips increases the feeding response in snails. An aversive stimulus (e.g. KCl) is used as the unconditional stimulus (US). Application of US to the snail inhibits feeding behavior. In CTA training, the CS is paired with the US. After repeated

temporal contingent presentations of the CS and US, the CS no longer elicits feeding response (i.e. the new behavioral response), and this aversive conditioning persists as a long-term memory (Kojima et al., 1996; Kawai et al., 2004). In *Lymnaea*, an identifiable neuron, known as the cerebral giant cell, has been hypothesized to be the key neuron for both the acquisition and formation of long-term memory of CTA (Kojima et al., 1997; Nakamura et al., 1999a; Kojima et al., 2001).

To begin to elucidate the cellular and molecular mechanisms underlying: (1) the acquisition of CTA, and (2) the consolidation of learning into long-term memory persistence, we have employed a number of different procedures utilizing techniques directed against specific molecules in snails (Kobayashi et al., 2000a; Kobayashi et al., 2000b; Hatakeyama et al., 2004a; Hatakeyama et al., 2004b; Hatakeyama et al., 2004c). For the formation and maintenance of long-term memory following CTA training, a molecular cascade is

necessary in the cerebral giant cells, involving cyclic AMP (cAMP), protein kinase A and cAMP responsive element binding protein (CREB) (Nakamura et al., 1999b; Ribeiro et al., 2003; Sadamoto et al., 2004a; Sadamoto et al., 2004b; Wagatsuma et al., 2005).

However, in our studies designed to determine how long the long-term memory for the CTA persists, it became apparent that snails continued to eat their normal diet of lettuce in their home aquaria while still having a memory for the CTA. Thus, it was unclear what the relationship was between CTA for a specific CS and other appetitive food stimuli. In other words, what capacity do snails have to differentiate between appetitive food stimuli during training and testing for long-term memory so that they can avoid one food type (i.e. CTA) whilst continuing to be attracted to another? Moreover, if they can successfully differentiate between appetitive food stimuli, where in the central nervous system does this occur? *Lymnaea* certainly have been shown to possess the capacity to distinguish between food types; in appetitive conditioning, they can differentiate between appetitive food stimuli (Straub et al., 2004), whereas in an operant conditioning procedure they can differentiate a carrot stimulus from other stimuli (Haney and Lukowiak, 2001; Sangha et al., 2005).

In the present study, we directly demonstrate that snails can be differentially conditioned to avoid one appetitive CS following CTA training whilst continuing to be responsive to another different appetitive food CS that has not been paired in a forward manner with an aversive US. In addition, we also found during the course of our studies that *Lymnaea* have the capability of undergoing second-order conditioning.

Materials and methods

Snails

Specimens of *Lymnaea stagnalis* L. with 20–25 mm shell lengths (i.e. young adults: Yamanaka et al., 1999; McComb et al., 2005b) were obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam supplemented with snails from the Calgary facility, which were also derived from the same Amsterdam colony). All snails were maintained in dechlorinated tapwater (i.e. pondwater) and fed lettuce on a 12 h:12 h L:D cycle at 20–24°C.

Conditioning procedures for conditioned taste aversion

Before conditioning, snails were starved for 1 day. Then, the snails were trained by a CTA procedure in a 60 mm Petri dish. The CS was either a 5 ml solution of 10 mmol l⁻¹ sucrose or a solution of 0.3% carrot juice, whereas the US was a 5 ml solution of 10 mmol l⁻¹ KCl. One of the CSs and the US were pipetted into a Petri dish for 15 s with a 15 s inter-stimulus interval. This pairing of CS and US was repeated 10 times with a 10 min inter-trial interval. Before and after the training session, a 5 ml solution of the CS was applied to the lip and washed out with distilled water. The feeding response elicited by the CS (i.e. bites min⁻¹) was determined for a 1-min interval. We compared the feeding response before training

(pre-test) to the feeding response following training (post-test). A backward-conditioned (the US precedes the presentation of the CS) control group and a naive (distilled water only applied to the lips instead of the CS and US) control group were also employed.

Conditioning procedures for second-order conditioning

We used the following training procedure to determine if *Lymnaea* were capable of undergoing second-order conditioning. The two appetitive CSs described above (sucrose and carrot juice) and the aversive US (KCl) were used. To determine if snails had the capacity to undergo second-order conditioning, we used a two-phase training procedure. In the first phase, snails were conditioned as previously described, and so the first-order CTA was established. Snails were conditioned to avoid one of the appetitive food substance (CS1) by pairing CS1 with the US. In the second phase, the other CS (CS2) was used and paired in a forward manner with the CS1, and so the CS1 now served as the US. If snails are capable of undergoing second-order conditioning, CS2 should no longer act as an appetitive stimulus, rather it acquires the ability to evoke CTA, even though it was never paired with the US (Carew and Sahley, 1986). In the present study, the CS1 (e.g. sucrose) was paired with the US 10 times, and then the CS2 (e.g. carrot juice) was paired with the CS1 10 times. The inter-stimulus interval and inter-trial interval were the same as those in the CTA training procedure first described above. We also performed the experiments using carrot juice as the CS1 and sucrose as the CS2.

The entire training and testing procedures were performed under a blind protocol. In the post-test, the good performers were defined as those snails responding to the CS with 0 or 1 bite min⁻¹; whereas the poor performers were those snails responding to the CS with 2 or more bites per minute.

Statistical analyses

Data are expressed as means ± s.e.m. Statistical significance ($P < 0.05$) was determined by one-way analysis of variance (ANOVA) followed by the *post hoc* Scheffé test and Student's *t*-test.

Results

Responses of naive snails to potential conditional stimuli

We first examined the dose-dependency of feeding responses (number of bites min⁻¹) to potential stimuli to be used as a CS in our experiments. Thus we tested sucrose (sweet), sodium L-glutamic acid (umami or savory) and carrot juice (complex) as potential CSs (Fig. 1). First using sucrose, we found that the elicited feeding response increased in a sigmoid fashion with the increasing concentration of D-sucrose (Fig. 1A). Next we found that whereas L-Glu induced a high feeding response at both 1 and 10 mmol l⁻¹, it did not evoke an increase in similar sigmoid fashion as did sucrose (Fig. 1B). Unexpectedly, combining sucrose with Glu failed to further increase the elicited feeding response (Fig. 1B). Finally, the

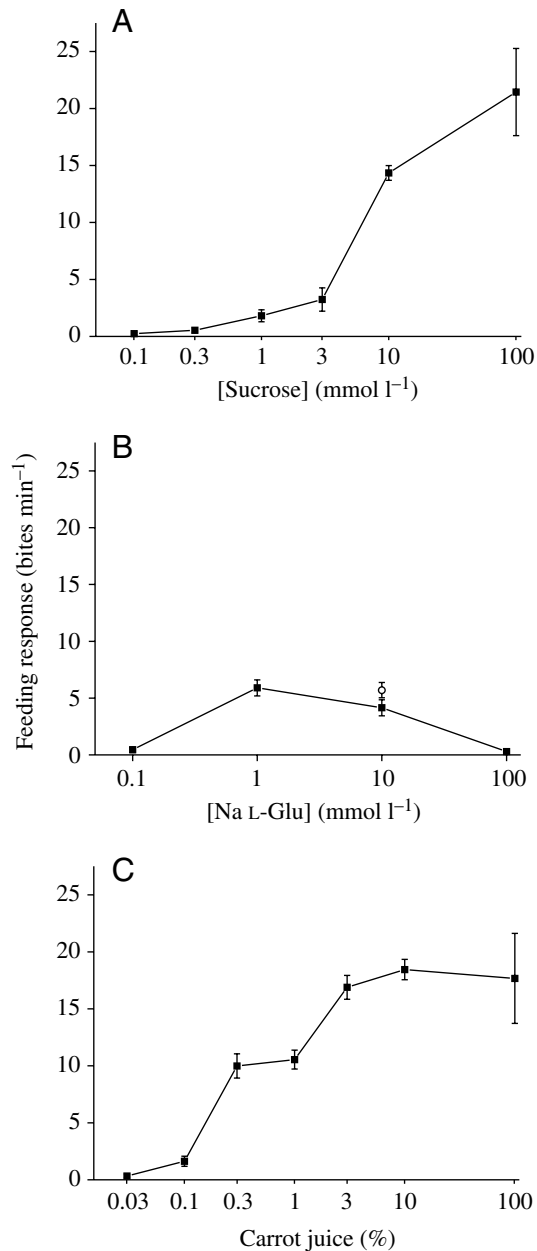


Fig. 1. Feeding responses elicited by different tastes applied to the lips of snails. (A) The number of bites min^{-1} elicited in snails by increasing the concentration of D-sucrose (sweet taste). (B) As in A, but sodium-L-glutamic acid (Na-L-Glu, umami taste, closed squares) was used as the stimulant. The open circles represent experiments in which a cocktail consisting of 3 mmol l^{-1} sucrose and 10 mmol l^{-1} Glu was applied to the lips. Notice that based on the data obtained in A, it would be expected that this cocktail would elicit a response of at least 10 bites min^{-1} . However, this cocktail only produced the same level of feeding as that produced by Glu alone or by sucrose alone at these concentrations. (C) Again as in A, but carrot juice (complex taste) was used as the stimulant to the lips. All data are means \pm s.e.m. obtained from 20 snails. The x axis is in logarithmic scale. These results show that 10 mmol l^{-1} sucrose or 0.3% carrot juice induces a reliable feeding response in snails.

could be used as the CS in these experiments. Thus the sucrose CS was paired with the US. Following CTA training, we found that the feeding response elicited by sucrose in the post-test session was significantly reduced ($P < 0.01$, one-way ANOVA followed by the *post hoc* Scheffé test) compared to both the pre-test session and to the post-test session of the backward-conditioning control or the naive control group (Fig. 2A). Moreover in all cases, there were no significant differences ($P > 0.05$) in the feeding responses elicited in the pre-test session of the CTA-trained cohorts, backward-conditioning control or naive control snails.

Similar results were obtained using carrot juice as a CS. CTA could be produced by the CTA-training procedure with carrot juice as the CS (Fig. 2B). We thus conclude that CTA can be learned in *Lymnaea* using either sucrose or carrot juice as a CS when paired in a forward but not backward manner.

Taste discrimination and memory retention in conditioned taste aversion

To directly determine if *Lymnaea* are capable of discriminating between the two appetitive stimuli (sucrose solution and carrot juice) used in CTA training, we first used the 10 mmol l^{-1} sucrose solution as the CS(+) and the 0.3% carrot juice as the CS(-). We then performed experiments using carrot juice as the CS(+) and sucrose as the CS(-). In all these experiments, both the CS(+) and the CS(-) were given to all snails. Moreover in the pre-test session (i.e. before any training), both the CS(+) and CS(-) induced a similar robust feeding response. However, after the CS(+) was paired 10 times with the US (the KCl stimulus), the CS(+) no longer elicited a robust feeding response. The feeding response elicited by the CS(+) in the post-test session given 48 h after training was significantly smaller than the response in the pre-test session ($P < 0.01$, Student's *t*-test, Fig. 3A). By contrast, the feeding response elicited by the CS(-) in the post-test session 48 h after training was statistically not different ($P > 0.05$) from the response elicited in the pre-test session (Fig. 3A).

Similar results were obtained when carrot juice was used as the CS(+) and sucrose as the CS(-) (Fig. 3B). The carrot juice no longer elicited a feeding response after training although

feeding response elicited by carrot juice also increased with its increasing concentration (Fig. 1C). Because both 10 mmol l^{-1} sucrose and 0.3% carrot juice always induce a reliable feeding response, we decided to use these two stimuli as our CSs in the following experiments to produce CTA.

Conditioned taste aversion experiments

Snails were trained on the basis of the 10-trial training (i.e. CS-US pairing) procedure described in the Materials and methods. As a CS, we used either 10 mmol l^{-1} sucrose or 0.3% carrot juice. A 10 mmol l^{-1} KCl solution was used as the US. We also prepared naive and backward-conditioned snails as controls. In all cohorts of snails, a pre-test to the specific CS (either sucrose or carrot juice) was given and the number of bites min^{-1} ascertained. We first examined whether sucrose

sucrose did. We thus conclude, as expected, that *Lymnaea* are capable of taste discrimination following CTA training and memory formation.

Second-order conditioning

Whereas second-order conditioning has been demonstrated previously in the terrestrial slug *Limax maximus* (Sahley et al., 1981), to our knowledge only the sensory preconditioning has been demonstrated in *Lymnaea* (Kojima et al., 1998). We therefore examined whether or not second-order conditioning is possible using two appetitive CSs (sucrose and carrot juice) described above. In the first series of experiments, we used the 10 mmol l⁻¹ sucrose solution as the CS1 and the 0.3% carrot juice as the CS2; the 10 mmol l⁻¹ KCl solution continued to be used as the US (Fig. 4A). In the first phase of training, the CS1 and the US were paired 10 times. In the second phase of training, the CS2 was then paired with the CS1 10 times.

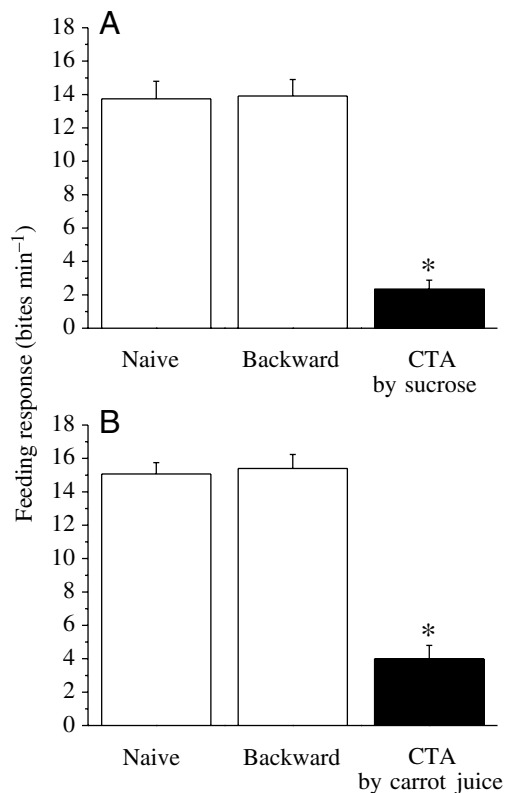


Fig. 2. Conditioned taste aversion (CTA) in snails. The numbers of bites min⁻¹ elicited by the CS [the conditional (taste) stimulus] in the test session following training. (A) Sucrose was used as the CS. The CS-elicited feeding response following training in CTA-trained snails ($N=40$) was significantly less ($*P<0.01$, one-way ANOVA followed by the *post hoc* Scheffé test) than in either snails given backward-conditioning ($N=35$) or naive ($N=39$) snails. Further, there was no significant difference in the elicited feeding response between the backward-conditioned group and the naive group. (B) Carrot juice was used as the CS. The data are plotted as in A, and similar results were obtained. The numbers of snails were as follows: 45 naive snails, 35 backward-conditioned snails, and 63 conditioned snails. All data are means \pm s.e.m.

We then tested whether or not the feeding response to CS2 (carrot juice) in the final test session was significantly reduced ($P<0.01$, Student's *t*-test) compared to the response elicited in the pre-test session. However, whereas carrot juice (CS2) elicited significantly fewer bites in the final test session than it did in the pre-test session, it still elicited significantly more bites ($P<0.01$, Student's *t*-test) than did sucrose (CS1). The CS1 thus suppressed the feeding response to a significantly greater extent than did the CS2 after the pairing of the CS2 with the CS1.

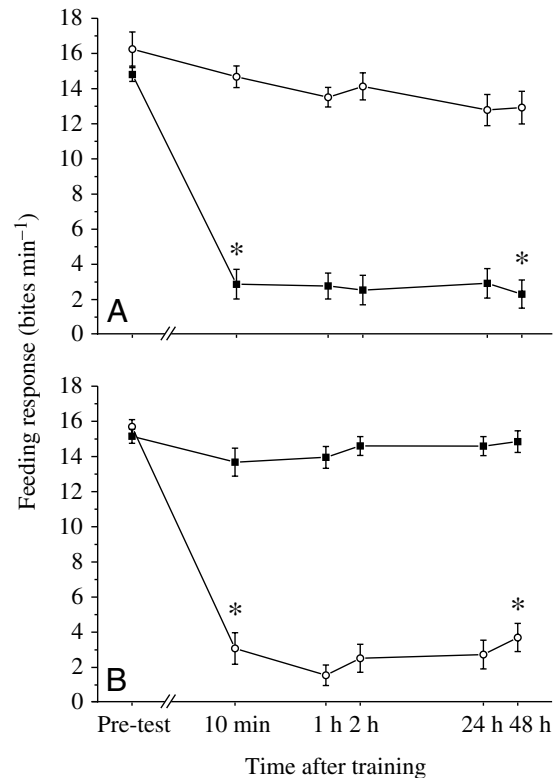


Fig. 3. Taste discrimination following conditioned taste aversion (CTA) in snails. (A) Sucrose was used as the conditional stimulus (CS) in the CTA training procedure. In the pre-test session, sucrose ($N=40$, closed squares) and carrot juice ($N=40$, open circles) elicited statistically similar feeding responses. At specific times following CTA training, sucrose elicited a significantly smaller feeding response than did carrot juice. Additionally, the feeding response elicited by sucrose at each time point following CTA was significantly less than it was in the pre-test session, whereas the feeding response elicited by carrot juice did not change significantly over the course of the experiment. The memory for CTA following this training procedure persisted for at least 48 h ($*P<0.01$, Student's *t*-test). The numbers of snails was reduced to 37 for carrot juice and 36 for sucrose at 48 h, because 3 and 4 snails, withdrew their bodies into their shells or died, respectively. (B) As in A, except that carrot juice was used as the CS. Similar results were obtained. All data are means \pm s.e.m. The numbers of snails became 38 for sucrose and 40 for carrot juice at 48 h because 2 snails for sucrose withdrew their bodies into the shells. The *x*-axes are logarithmic scale. Thus snails are able to taste discriminate following CTA learning, memory formation and memory persistence.

We obtained statistically similar results when we used carrot juice as the CS1 and sucrose as the CS2 (Fig. 4B). We can therefore draw two conclusions from these data. First, second-order conditioning is possible in *Lymnaea*. Second, even after second-order conditioning, snails are still capable of discriminating between the two CSs.

Extinction of conditioned taste aversion memory

Whereas extinction of a learned and remembered operantly conditioned behavior in *Lymnaea* has previously been demonstrated (Sangha et al., 2003), to our knowledge extinction of a learned and remembered CTA has not been demonstrated. We therefore attempted to extinguish CTA. To determine if we could extinguish a CTA, we first trained snails as described. We thus paired the sucrose CS with the KCl US, and this produced CTA (Fig. 5A). Following the learning, we presented the CS alone (i.e. it was not paired with the US) three times at 10 min intervals (i.e. 10 min, 20 min and 30 min after

the end of conditioning procedure). At each presentation of the CS alone (equivalent to a memory test), we measured the number of bites elicited by the CS.

As can be seen, the memory for CTA was not extinguished. The number of bites elicited by the sucrose CS following the attempted extinction procedure was still significantly less ($P < 0.01$, Student's *t*-test) in each of the CS alone sessions and also in each of the post-test sessions (up to 24 h after training). In addition, even when we separated out the so-called good and poor performers (see Materials and methods), extinction did not occur. Moreover, the difference between the feeding responses elicited by the CS following training and attempted extinction between good and poor performers continued to be maintained.

Finally, when carrot juice was used as the CS (Fig. 5B), the data we obtained were similar to those obtained with sucrose. Thus, we conclude that the CTA memory is not extinguished by the repetitive conditional stimuli.

Discussion

In the course of examining the specific question of whether *Lymnaea* have the capability to discriminate between different food tastes after having acquired and committed to memory CTA, we found: (1) CTA for a specific food taste does not generalize to other tastes; (2) long-term memory for CTA is difficult to extinguish; and (3) *Lymnaea* are capable of undergoing second-order conditioning for CTA. Each of these findings has previously been an unexplored aspect of CTA in the *Lymnaea* model system. The importance of whether or not *Lymnaea* are able to discriminate between different food tastes

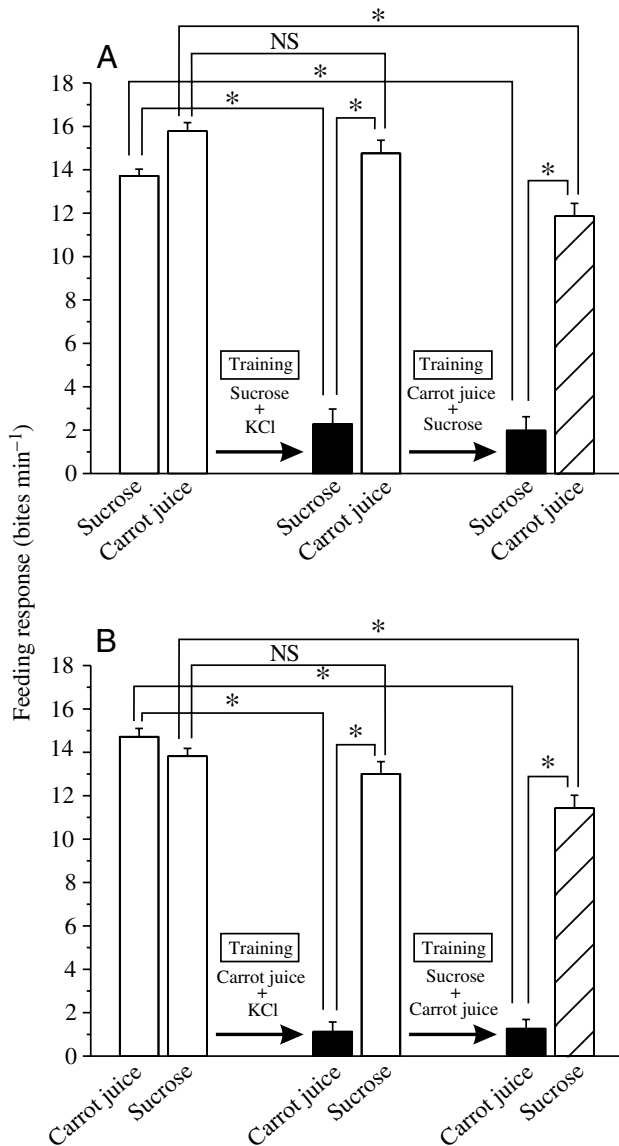


Fig. 4. Second-order conditioning of feeding behavior in snails. (A) In the pre-test session, sucrose (CS1; conditional stimulus 1) and carrot juice (CS2) elicit similar feeding responses in naive snails ($N=50$). Conditioned taste aversion (CTA) training with sucrose as the CS (CS1) and KCl as the US was then performed. In the first post-test session, the CS1 elicited a significantly smaller feeding response whereas the CS2 (carrot juice) elicited a similar response as in the pre-test session. In the first post-test session, the response elicited by sucrose (CS1) was also significantly less ($*P < 0.01$, Student's *t*-test) than that elicited by the CS2. In the next phase of training, the CS2 (carrot juice) was paired with the CS1 (sucrose). Following this training, a second post-test session was performed. In this session, the feeding response elicited by CS2 (carrot juice) was significantly less ($*P < 0.01$, Student's *t*-test) than the response it elicited in either the pre-test session. Notice, however, that the response elicited by CS2 was still significantly greater ($*P < 0.01$, Student's *t*-test) than the response elicited by CS1. The number of snails was reduced to 49 after the first-phase training because one snail withdrew its body into its shell, and then 50 snails were used for the second-phase training. (B) As in A ($N=50$), except that carrot juice was the CS1 and sucrose the CS2. Identical results were obtained as in A. The numbers of snails was reduced to 48 and 49 after the first-phase training because 2 and 1 snail withdrew their bodies into their shells for the CS1 and the CS2, respectively, and then 49 snails were used for each of the second-phase training. All data are means \pm s.e.m.

after the acquisition of CTA is that it directs future studies to determine both where and how in the central nervous system changes occur that are causative for learning and memory formation and its maintenance. The additional two findings will: (1) allow us to design further neural and molecular experiments to explore why CTA memory is resistant to both extinction and forgetting and (2) how second-order conditioning occurs at both the neuronal and molecular levels in *Lymnaea*.

Taste discrimination system existing before memory formation

We first showed that different food tastes elicit different levels of feeding, as assessed by the number of bites elicited by the food taste to the lip area of the snail (Fig. 1). For both sucrose and carrot juice, there was the expected dose–response curve with increasing concentrations of the food substance eliciting a larger feeding response. In contrast, the response elicited by glutamate was different in two respects. First, glutamate did not elicit a robust feeding response, as compared to both sucrose and carrot juice, and second its dose–response was a U-shaped curve. Higher concentrations actually elicited a weaker feeding response. Puzzling also was the fact that the combination of glutamate and sucrose did not result in an additive response.

Where and how (i.e. the neural basis of food discrimination) taste discrimination occurs is not certain in *Lymnaea*, although a recent paper (Straub et al., 2004) presents compelling data that the output from peripheral sensory neurons in the lip area is not significantly altered following appetitive conditioning, suggesting that taste discrimination does not occur peripherally. Future experiments will have to address this question. The results from these initial experiments allowed us to choose appropriate concentrations of different food stimuli that elicit comparable levels of feeding in naive snails. Thus we were able to test taste discrimination following CTA training.

Having shown that two different tastes elicited robust feeding responses, we had to ascertain whether or not each specific taste could undergo CTA. We found that CTA produced by pairing either sucrose or carrot juice with the suppressive KCl stimulus was similar (Fig. 2). Previously, our group had only used sucrose for CTA (Kojima et al., 1996; Kojima et al., 1997; Wagatsuma et al., 2004). Thus *Lymnaea* can learn and remember to avoid different food tastes. Whereas similar findings were found previously in *Limax* (Sahley et al., 1981), this is the first time it has been demonstrated in our *Lymnaea* model system.

After acquiring and committing to memory a CTA for a specific food taste, we found that snails have the capacity to discriminate between that taste and a different taste (Fig. 3). The feeding response elicited by the safe taste (i.e. the taste not paired with the KCl aversive stimulus) in CTA-trained snails was similar to that elicited by the taste in naive snails. Either of the two tastes we employed in our experiments could serve as the conditioned or the safe taste. Thus, CTA training did not appear to alter the ability of a safe taste to elicit a feeding response.

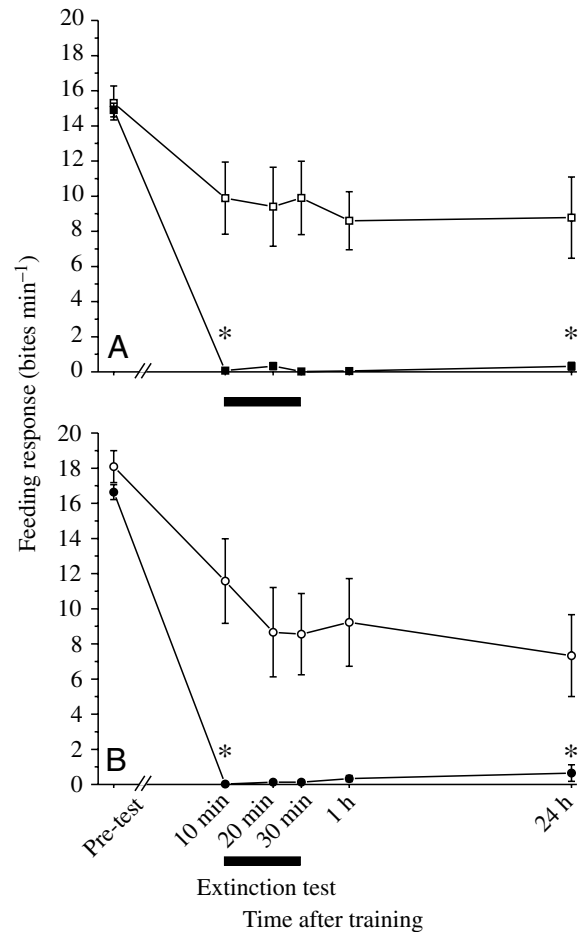


Fig. 5. Extinction and conditioned taste aversion memory. (A) Naive snails ($N=50$) were first challenged with the sucrose taste. This elicited robust feeding. Snails were then conditioned taste aversion (CTA) trained. 10 min after training, snails were given the conditional stimulus (CS) alone (i.e. an extinction session) and the elicited feeding response noted. Snails were designated as either good ($N=40$; closed boxes) or poor ($N=10$; open boxes) performers. All snails received two more (at 10 min intervals) extinction sessions. Again in each extinction session the elicited feeding response was noted. All snails were then challenged with the sucrose stimulus 1 and 24 h later. Extinction did not occur. The three extinction sessions did not result in a behavioral phenotype resembling the naive state in either the good or the poor performers. (B) As in A, except carrot juice was used as the CS. Similar results were obtained. All data are means \pm s.e.m. The differences between the feeding response of the good performers and that of the poor performers in the both cases were maintained for at least 24 h with $*P<0.01$ (Student's *t*-test), showing that the memory formed by CTA training cannot be extinguished.

These results suggest that the loci for taste discrimination occur upstream from the site of CTA memory formation and storage. Previously, Kojima et al. (Kojima et al., 1997; Kojima et al., 2001) proposed a neuronal model for CTA. In their model the cerebral giant cells played a pivotal role in CTA. Activity in the cerebral giant cells was hypothesized to suppress the activity of the central pattern generator that drives feeding behavior. This suppression was thought to be due to

the enhancement of inhibitory postsynaptic potentials from the cerebral giant cells to the central pattern generator that drives feeding in the conditioned snails (Kojima et al., 1997; Kojima et al., 2001). The data that we have obtained here, namely the ability to discriminate food tastes after CTA, are not consistent with the predictions of the Kojima model. As the model was presented following CTA, a different food taste should not have been able to elicit a feeding response since for all effective purposes, the central pattern generator that drives feeding behavior could not have been activated according to the Kojima model. Clearly as our data show this is not the case. Thus we must now modify the Kojima model.

However, the necessity of modifying the Kojima model does not mean that the cerebral giant cells play no role or only a minor role in mediating CTA learning and memory. What the results presented here mean is that the circuits underlying learning and memory formation are much more complicated than we initially envisioned. This notion that the neural basis of learning and remembering various feeding behavior is more complicated than initially thought is not only our view, but it is the view of others working on feeding in *Lymnaea*. For example, Straub and his colleagues showed that no change was observed in the peripheral inputs following the appetitive taste conditioning to a food taste that initially did not activate feeding behavior in a robust manner (Straub et al., 2004). Their data suggest that the changes occurring at loci not yet identified probably mediate the taste discrimination. Our data presented here are consistent with the view expressed by Straub et al. (Straub et al., 2004). Thus, we must consider a mechanism for taste discrimination in the neural pathways before the cerebral giant cells in the central nervous system.

Second-order conditioning by tastes

In the course of our experiments to show taste discrimination following CTA, we found that it is possible to induce second-order conditioning in *Lymnaea*. To our knowledge this is the first instance that second-order conditioning has been demonstrated in *Lymnaea*. Previously, this form of conditioning was demonstrated in another molluscan preparation *Limax* (Sahley et al., 1981). We are not sure if any other group has attempted to show such higher order conditioning in other molluscan preparations. It is our feeling that it would be possible to do so, but most probably it has not been tried. An advantage that the *Lymnaea* model system may have over the *Limax* model system is that the *Lymnaea* neural circuitry may be a little better analyzed (e.g. Ito et al., 2003a; Ito et al., 2003b; Ito et al., 2004; Fujie et al., 2005). What the ability to demonstrate second-order conditioning shows is that CTA is an extremely robust memory, in that the memory for one taste that initially elicited feeding response can be used following CTA as a negative reinforcer for a second appetitive taste. This again confirms the extreme robustness of the CTA memory.

Rigid memory formed by conditioned taste aversion training

Extinction as famously described in early work by I. P. Pavlov is the repeated exposure to the CS (in our case a food

taste) without the presentation of the US (the KCl stimulus) (Mackintosh, 1974). If extinction occurs the conditioned response (i.e. CTA) is decreased. Importantly, extinction training has been shown to be the result of new memory formation and does not erase the previously learned response (Bouton, 1993). Extinction is not unlearning, as the original memory can be shown to still be present (i.e. the phenomenon of spontaneous recovery). There is evidence that competition may exist between the original memory and the extinction memory (Sangha et al., 2005). It is not clear what mechanism(s) allows one memory to dominate over the other.

Here we demonstrated that the CTA memory was also resistant to extinction (Fig. 5). This implies that the CTA memory is robust. A robust and long-lasting memory is a hallmark of CTA. In mammals, for example, CTA has been associated with gut illness (Garcia et al., 1955), viewed as part of a gut defense system (Garcia et al., 1985), and formed quickly and maintained as long-term memory (Garcia et al., 1974). Thus, it is not too surprising that this memory is resistant to extinction training. Further experiments will be necessary to determine what the molecular basis of CTA memory is in *Lymnaea* (e.g. Sadamoto et al., 2004a; Sadamoto et al., 2004b; Wagatsuma et al., 2005).

These data and consideration of the mammalian data suggest to us that we need to incorporate feedback from the gastric system into our model in order to consolidate the memory in the central nervous system in *Lymnaea*. Because the B2 motoneurons, which control digestion, can release nitric oxide and affect the buccal neurons (Sadamoto et al., 1998; Kobayashi et al., 2000a; Kobayashi et al., 2000b), the B2 motoneurons may associate gastric malaise with inhibition of feeding behavior, resulting in consolidation of the CTA memory (Hatakeyama et al., 2006). Future experiments will be designed to specifically test this hypothesis. Interestingly, nitric oxide is involved in odor discrimination in the associative learning of another mollusc, *Limax* (Sakura et al., 2004).

In conclusion, *Lymnaea* can distinguish between tastes following CTA. The neurons responsible for taste discrimination may be located in the central nervous system and most probably occur upstream of the cerebral giant cells. It is also possible to employ the ability of *Lymnaea* to show taste discrimination following CTA to bring about second-order conditioning. Finally, together with the results showing that extinction of the CTA is difficult, we are led to the conclusion that the engram of CTA memory is well entrenched in neurons in the central nervous system. This may allow us to more easily employ techniques to uncover the cascade of events that lead to both the establishment and maintenance of long-term memory at the level of single neurons.

This work was supported in part by Grants-in-Aid (Nos. 16370033 and 17657049) from the Japan Society for the Promotion of Science to E.I., a fund from the Canadian Institutes of Health Research to K.L., and a fund from the Goho Life Sciences International Fund to K.L. and E.I.

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