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One-trial conditioned taste aversion in *Lymnaea*: good and poor performers in long-term memory acquisition

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Summary

In the majority of studies designed to elucidate the causal mechanisms of memory formation, certain members of the experimental cohort, even though subjected to exactly the same conditioning procedures, remember significantly better than others, whereas others show little or no long-term memory (LTM) formation. To begin to address the question of why this phenomenon occurs and thereby help clarify the causal mechanism of LTM formation, we used a conditioned taste aversion (CTA) procedure on individuals of the pond snail *Lymnaea stagnalis* and analyzed their subsequent behavior. Using sucrose as an appetitive stimulus and KCl as an aversive stimulus, we obtained a constant ratio of 'poor' to 'good' performers for CTA–LTM. We found that approximately 40% of trained snails possessed LTM following a one-trial conditioning procedure. When we examined the time-window necessary for the memory consolidation, we found that if we cooled snails to 4°C for 30 min within 10 min after the one-trial conditioning, LTM was blocked. However, with delayed cooling (i.e. longer than 10 min), LTM was present. We could further interfere with LTM formation by inducing inhibitory learning (i.e. backward conditioning) after the one-trial conditioning. Finally, we examined whether we could motivate snails to acquire LTM by depriving them of food for 5 days before the one-trial conditioning. Food-deprived snails, however, failed to exhibit LTM following the one-trial conditioning. These results will help us begin to clarify why some individuals are better at learning and forming memory for specific tasks at the neuronal level.

Key words: cooling, long-term memory, motivation, one-trial conditioning.

Introduction

Lymnaea stagnalis is an excellent model molluscan system to use in elucidating the causal neuronal and molecular mechanisms underlying both the associative acquisition of a new behavior (i.e. learning) and its subsequent consolidation into long-term memory (LTM) (Ito et al., 1999; Benjamin et al., 2000; Lukowiak et al., 2003; Sakakibara, 2006). For example, *Lymnaea* exhibit appetitive (Kemenes, G. et al., 2006) or avoidance (Sakakibara et al., 2005) classical conditioning; as well as operant conditioning of either their aerial respiratory behavior (Parvez et al., 2006; Lowe and Spencer, 2006) or their escape response (Kobayashi et al., 1998). Additionally, it is possible to use a one-trial conditioning procedure that results in LTM in this model (Alexander et al., 1984; Kemenes et al., 2002; Kemenes, I. et al., 2006; Fulton et al., 2005).

Typically investigators attempt to optimize the conditioning procedures to produce the most robust learning and subsequent LTM formation and thereby enhance their chances of uncovering causal neuronal mechanisms of LTM formation. However, in most cohorts of experimental subjects, there is a subset of subjects that performs significantly better (or significantly worse) than the others in constructing LTM (e.g. Alkon et al., 1990; Ito et al., 1994; Spencer et al., 1999; Lukowiak et al., 2003; Rosenegger et al., 2004). The cause of this phenomenon is unclear.

The phenomenon of a subset of animals not forming LTM also occurs in the conditioning procedures used to produce conditioned taste aversion (CTA) in both *Lymnaea* (Kojima et al., 1996; Kawai et al., 2004) and mammals (Masugi et al., 1999). Conventional wisdom has had it that LTM following CTA occurs in 100% of animals used, but this is not the case (Garcia et al., 1955; Garcia et al., 1974; Garcia et al., 1985). However,

by studying why some animals do not form LTM following the one-trial CTA procedure, it may be possible to elucidate the causal mechanisms of memory formation following the CTA procedure, much as in the studies using mutant strains of *Drosophila* and *C. elegans* (Dubnau and Tully, 1998; Waddell and Quinn, 2001; Margulies et al., 2005; Rankin, 2005).

In our previous experiments examining CTA as well as in the present study, sucrose or carrot juice, both of which increase the feeding response, was used as the conditioned stimulus (CS), whereas KCl, which inhibits feeding behavior, was used as the unconditioned stimulus (US) (Kojima et al., 1996; Sugai et al., 2006). Following the paired presentations of the CS and US, sucrose or carrot juice no longer acts as an appetitive stimulus, and this CTA persists as an LTM for more than a month (Kojima et al., 1996). It has been hypothesized that the cerebral giant cells and the B2 motor neurons play key roles in mediating the learning and LTM formation of this CTA (Kojima et al., 1997; Kojima et al., 2000; Kojima et al., 2001; Hatakeyama et al., 2004a; Sadamoto et al., 2004b). Consistent with this hypothesis are the data showing that altered gene activity and new protein synthesis must occur in these and other neurons if LTM is to form following the CTA procedure (Sadamoto et al., 2004a; Hatakeyama et al., 2004b; Hatakeyama et al., 2006; Azami et al., 2006; Wagatsuma et al., 2006). In addition, our recent findings showed that snails are both able to discriminate different tastes and continue to eat other foods following the CTA procedure (Sugai et al., 2006).

As a first step in attempting to determine why some snails form LTM and others do not, we have been concentrating our efforts on experiments designed to show that the differences between 'good' and 'poor' performers (i.e. those that have memory *versus* those that do not) are consistently seen, and we then compared the behavioral features that differentiate between good and poor performers. In the present study, we thus applied the one-trial CTA procedure in *Lymnaea*. Our results showed that whereas it is easy to differentiate between good and poor performers after conditioning, as of yet there is no easily determinable difference in behavioral phenotype between the two groups before conditioning.

Materials and methods

Snails

Specimens of *Lymnaea stagnalis* L. with shell lengths of 20–25 mm (i.e. young adults) (Sadamoto et al., 2000) were obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam, supplemented with snails from the Calgary facility that were also derived from the same Amsterdam colony). All snails were maintained in de-chlorinated tapwater (i.e. pond water) and fed lettuce under a 12 h:12 h light:dark cycle at 18–22°C.

Conditioning and control procedures for conditioned taste aversion

All snails were deprived of food for 1 day before conditioning. Data from pilot experiments demonstrated that 1 day food deprivation before the CTA conditioning procedure was necessary to obtain consistent results. Depriving snails of food for 1 day did not appear to alter such easily observable behaviors as egg laying or aerial respiration (personal observations), and did not result in any change in the general health of snails, such as evidenced by a change in mortality rate. In other experiments, as described below, snails were deprived of food for 5 days before the CTA procedure.

Snails were conditioned for CTA in a 60 mm (diameter) Petri dish. The CS used was either 5 ml of 10 mmol l^{-1} sucrose solution (CS1) or 5 ml of 0.3% carrot juice solution (CS2), whereas the US was 5 ml of 10 mmol l^{-1} KCl. Either CS elicits a reliable feeding response (number of bites min^{-1}) from snails deprived of food for 1 day, whereas the US reliably causes the snails to stop their feeding behavior (Sugai et al., 2006). Higher concentrations of KCl cause snails to withdraw into their shells for a long period (Kojima et al., 1996) and so were not used in our studies. Snails were placed in the Petri dish and then either CS1 or CS2 was poured into the Petri dish; 15 s later the solution was exchanged for US (i.e. the inter-stimulus interval was 15 s). All conditioning and testing procedures described above were performed using a blind protocol.

One-trial conditioning procedure

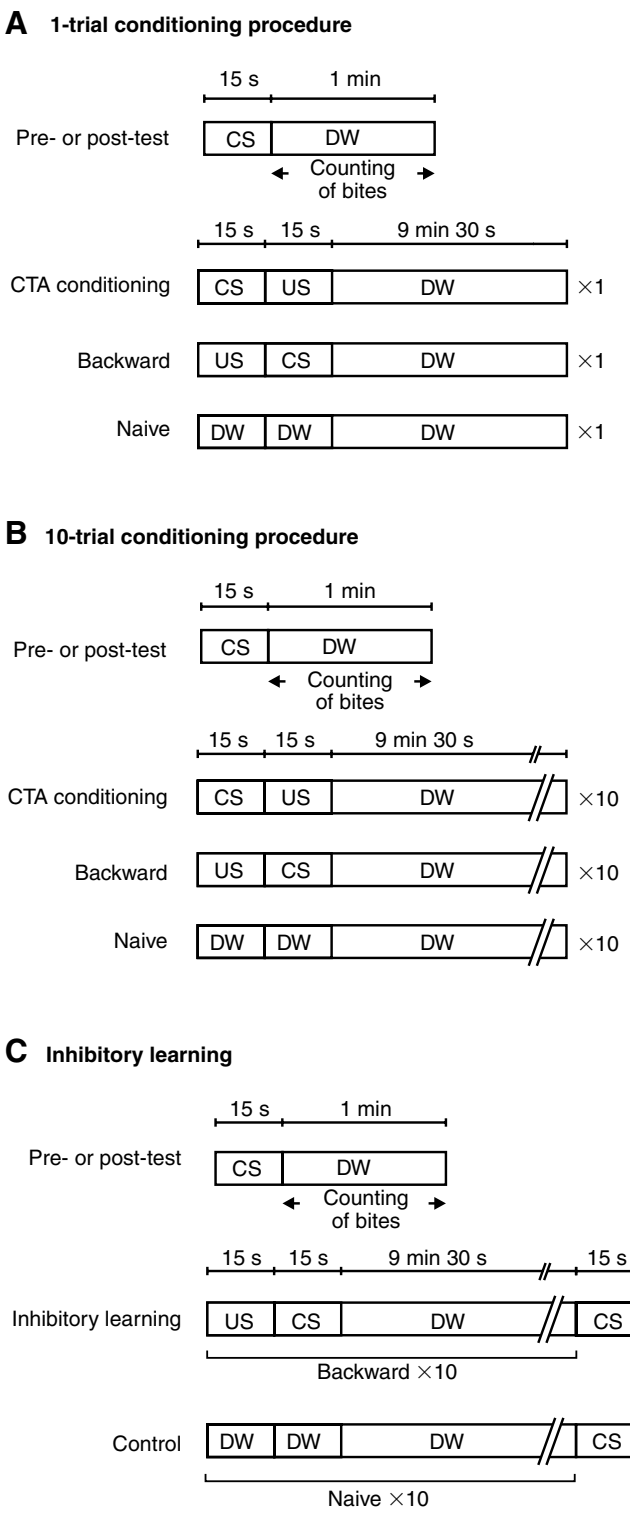
All snails were first given a pre-test session to determine their feeding response to the CS. If the CS did not elicit an adequate feeding response ($\geqslant$10 bites min^{-1}) the snail was not used in the learning and memory experiments. In the pre-test session snails were given either CS for 15 s, and then the CS was rinsed from the Petri dish with distilled water (DW). The number of bites made by the snails in DW was counted over the course of the next 1 min. The pre-test session was given to the snails at least 10 min before the one-trial conditioning.

In the one-trial CTA procedure, snails were first exposed to the CS for 15 s (Fig. 1A). The CS was then rinsed out and was followed by a 15 s exposure to the US. The change of solutions was done using a micropipette. A post-test session identical to the pre-test one was then performed at the specified times following the paired presentation of the CS–US.

Two different control experiments were performed to demonstrate that the significant change in feeding behavior in the post-test session was the result of an association between the CS and US. In the first control experiment, referred to as the backward-conditioning control, the temporal presentation of the CS and US was reversed. That is, snails were first exposed to the US for 15 s and then exposed to the CS for a further 15 s. These snails were given the post-test session at the times specified. In the second control experiment, referred to as the naive control, snails were exposed to only DW. The DW was poured over the snails at exactly the same time and interval as the CS and US in the other two cohorts. The naive-control snails were given both the pre- and the post-test session to the CS.

Ten-trial conditioning procedure

Snails deprived of food for 1 day were first given the same pre-test session as described above at least 10 min before the



10-trial conditioning. The conditioning procedure, the CS paired with the US, was the same as for the one-trial conditioning procedure except in this conditioning procedure the CS–US pairing was performed 10 times with a 10 min inter-trial interval (Fig. 1B). The snails were then given the post-test conditioning session at the time specified. Two control cohorts were also run. The backward-conditioning control cohort was treated in exactly the same manner as the experimental cohort, except that the US preceded the CS by 15 s. The naive-control cohort received only the DW stimulus.

Cooling

In *Lymnaea*, we can use a quickly applicable, reversible, benign amnesiac agent termed cold-block. Experiments on the operant conditioning of aerial respiratory behavior have shown that placing snails in water cooled to 4°C prevents LTM formation (Sangha et al., 2003b). We thus applied this to our snails immediately following the CTA procedure. Just after termination of the conditioning, the snails were placed into cooled 4°C DW and were maintained at this temperature for 30 min. Following this 30 min cooling, snails were placed into room temperature (20°C) DW for at least 20 min before the post-test session.

Inhibitory learning

As above, all snails were first given the pre-test session at least 10 min before the inhibitory learning. Following the pre-test session, snails were subjected to 10 backward-conditioning sessions as described above (Fig. 1C). Snails were then given the one-trial conditioning session, again, as described above. The interval between the last backward-conditioning session and the one-trial conditioning session was 10 min. Memory was assessed in the post-test session at the stated intervals following the one-trial conditioning session. As a control for inhibitory learning, snails were subjected to repeated DW sessions instead of the backward-conditioning sessions before they received the one-trial conditioning session.

Fig. 1. Conditioning procedures for conditioned taste aversion (CTA) in snails. (A) The one-trial conditioning procedure. (B) The 10-trial conditioning procedure. The conditioned stimulus (CS) was 5 ml of either 10 mmol l^{-1} sucrose solution or 0.3% carrot juice, and the unconditioned stimulus (US) in all cases was 5 ml of 10 mmol l^{-1} KCl solution. The CS and US were added to the Petri dish using a pipette for 15 s with a 15 s inter-stimulus interval. The inter-trial interval was 10 min in the 10-trial conditioning procedure. Before and after the conditioning procedure, the CS was applied to the lips and washed off with distilled water. Then, the feeding response was determined for 1 min as a pre-test or a post-test. A backward-conditioning (US–CS) control procedure and a naive (presented only with distilled water) control procedure were also employed. (C) Inhibitory leaning. After a 10-trial backward-conditioning procedure, a normal one-trial CTA conditioning procedure was used. As a control, the naive procedure was employed instead of the backward-conditioning procedure. We also used two different CSs, CS1 (10 mmol l^{-1} sucrose) and CS2 (0.3% carrot juice), and one US (10 mmol l^{-1} KCl). All the conditioning procedures were performed with a blind protocol.

Operational definition of good and poor performers

Based on data from pilot experiments, we set a performance boundary to distinguish between 'good' and 'poor' performers. A snail possessing LTM (i.e. a good performer) is expected not to open its mouth following presentation of the CS. However, some snails open their mouths by chance (i.e. spontaneously) in the absence of any delivered stimulus (Kojima et al., 1996). Such spontaneous openings occur at a rate of about one per min. Thus, we defined a good performer as a snail that made 0–1 bites min^{-1} during the post-test session in response to the CS. Poor performers were thus defined as snails that made $\geqslant$2 bites min^{-1} in response to the CS during the post-test session.

Statistical analyses

Data are expressed as the means ± s.e.m. Statistical significance (P<0.05) was determined by one-way analysis of variance (ANOVA) followed by the *post-hoc* Scheffé's test. The Student's *t*-test was also used to determine significant differences between two cohorts.

Results

One-trial conditioning for conditioned taste aversion and its long-term memory

A cohort of 50 snails that were randomly collected from the water tank was initially used to determine if our one-trial conditioning procedure was able to produce LTM. In this first experiment, 10 mmol l^{-1} sucrose was used as the CS and 10 mmol l^{-1} KCl as the US. All 50 snails showed a feeding response to the CS in the pre-test session (17.2±0.5 bites min^{-1}). These snails were then subjected to the one-trial CTA procedure (Fig. 1A). We examined the feeding response of the snails in the post-test session 9 min and 30 s after the termination of the conditioning session. We found that 21 of the 50 snails (42%) met the criterion to be classified as good performers whereas 29 (58%) did not, and were thus classified as poor performers (Fig. 2A). That is, the CS elicited a feeding response of either 0 or 1 bite min^{-1} in 21 of the 50 snails in the post-test session (0.1±0.1 bites min^{-1}). In the pre-test session, these 21 snails had a feeding response to the CS of 17.0±0.8 bites min^{-1}. On the other hand, in the 29 snails that were classified as poor performers, the CS elicited 17.3±0.7 bites min^{-1} in the pre-test session and 17.4± 1.0 bites min^{-1} in the post-test session (Fig. 2B).

We then determined whether there was a significant difference in the number of bites evoked by the CS in the post-test session (10 min after the one-trial conditioning session) compared to the pre-test session in both the good and poor performers (Fig. 2B). In the good performers we found that there was a significant decrease in the feeding response in the post-test session compared to the pre-test session (P<0.01; Student's *t*-test). However, in the poor performers there was no significant difference in the feeding response elicited by the CS between the pre- and post-test sessions (P>0.05). Moreover, the feeding response elicited by the CS in the post-test session of

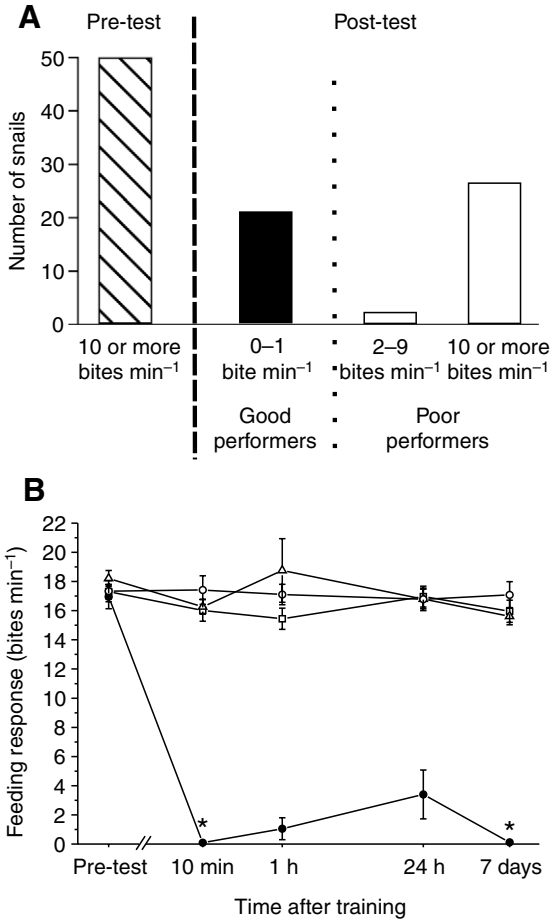


Fig. 2. Conditioned taste aversion following a one-trial conditioning procedure in snails using sucrose as the conditioned stimulus (CS) and KCl as the unconditioned stimulus (US). (A) The ratio of good and poor performers is assessed by their response to the CS following the one-trial conditioning procedure. We defined good performers as those snails that significantly reduced their response to the CS following the one-trial conditioning procedure. This meant that good performers reduced the number of bites min^{-1} in response to the CS from a level of approximately 17 in the pre-test to between 0 and 1 in the post-test session. In poor performers, however, there was no significant difference in the response to the CS in the post-test session compared to the pre-test session. Thus, in each session snails responded to the CS with approximately 17 bites min^{-1}. We found that 21 of 50 snails could be classified as good performers. That is, about 40% of snails acquired conditioned taste aversion (CTA). (B) The persistence of memory following the one-trial conditioning procedure. The numbers of snails at the pre-test were as follows: 50 for naive snails (open triangles), 50 for backward-conditioning snails (open squares), 21 for good performers (solid circles) and 29 for poor performers (open circles). All values are means ± s.e.m. The *x*-axis is expressed in a logarithmic scale. The difference between the feeding response of the good performers and that of the poor performers and control snails was maintained for at least 7 days at *P<0.01 (one-way ANOVA followed by the *post-hoc* Scheffé's test). The numbers of good performers and poor performers became 15 and 23, respectively, at 7 day because some snails withdrew their body into the shell or had died.

the good performers was significantly less than response of the poor performers ($P<0.01$). Finally, there was no significant difference in the feeding response elicited by the CS between good and poor performers in the pre-session ($P>0.05$). Thus, we conclude that following our newly developed one-trial conditioning procedure, there is a significant change in the behavior of a subset of snails that we have labeled as good performers.

We next determined whether the significant change observed in feeding behavior in the good performers as a result of the one-trial conditioning procedure was a *bona fide* example of associative learning. It is possible that the significant decrease in elicited feeding response by the CS in the post-test session of these snails was due to repeated stimulation of the snails by solution changes or that the decrease in feeding response was the result of a non-associative effect of the exposure to the aversive KCl stimulus. We therefore subjected another two cohorts of snails in our snail-rearing facility to one of the following control procedures: (1) snails in the Petri dish received the CS and US in altered sequence (i.e. a backward-conditioning control procedure in which the US preceded the CS); and (2) snails in the Petri dish were only exposed to distilled water (DW; referred to as the naive control procedure). In neither control cohort was there a significant difference in the feeding response elicited by the CS between the pre- and post-test sessions (i.e. $P>0.05$ for all tests), nor did any of these snails meet the good performer criterion (Fig. 2B). Moreover, there was also no significant difference in the response elicited by the CS in the post-test session when we compared the backward-conditioning control cohort, the naive control cohort and the poor performers that received the one-trial conditioning procedure (Fig. 2B). Thus we conclude that the change in feeding behavior observed in the post-test session of the good performers was the result of associative learning and the consolidation of the learning into memory.

Finally, we retrospectively examined whether good performers responded differently to the US than poor performers. The responses of all snails to the US were recorded before we knew how each snail would respond in the post-test session. All snails immediately stopped eating and moving around as soon as they received the US. Furthermore, we could not discern any difference between the two groups with respect to how soon they began to locomote following the presentation of the US (data not shown).

The above data demonstrate that following the one-trial conditioning procedure there is memory in a subset of the snails. However, the above experiment does not demonstrate that the memory is LTM (i.e. persisting for at least 24 h). We, therefore, tested a subset of the good ($N=15$) and poor ($N=23$) performers 7 days after the one-trial conditioning procedure. As was apparent (Fig. 2B), memory was still present 7 days after the one-trial conditioning session in the good performers and not present in the poor performers; this in spite of the fact that all snails were tested 10 min after the conditioning session in the absence of any reinforcement. That is, in the good performers, the feeding response (number of bites min^{-1}) elicited by the CS was significantly less in the post-test session than in the pre-test session 7 days before ($P<0.01$). By contrast, in the poor performers there was no significant difference in the feeding response to the CS in the 7-day post-test session compared to the pre-test session ($P>0.05$). We further compared the feeding response elicited by the CS in the 7-day post-test session between the good and poor performers and found that the response of the good performers was significantly less than the response in the poor performers ($P<0.01$; Fig. 2B).

Finally, when we tested snails in the backward-conditioning control and naive control cohorts 7 days later, we found that their response to the CS was not significantly different than that in the pre-test session ($P>0.05$ for both groups), and that in both cohorts the response was significantly greater than the feeding response elicited by the CS in the post-test session of the good performers ($P<0.01$, one-way ANOVA followed by the *post-hoc* Scheffé's test; Fig. 2B). We, therefore, conclude that memory following the one-trial conditioning procedure persists for at least 7 days and can therefore be classified as LTM.

Extinction of the conditioned taste aversion

Extinction is new learning and memory that occludes the old memory but does not annihilate it (Mackintosh, 1974; Bouton, 1993). It is also much more difficult to cause extinction of a strong memory than a weaker memory (Sangha et al., 2003a). We have previously shown that the phenomenon of extinction did not occur in snails trained by the 10-trial conditioning procedure for CTA (Sugai et al., 2006). We, therefore, wished to determine, as a second means of assessing LTM strength, whether the LTM following our newly developed one-trial CTA procedure was also resistant to extinction.

Two 50-snail cohorts randomly collected from the water tank were first given a pre-test session. The first cohort was exposed to sucrose as the CS (Fig. 3A). This cohort was then given the appropriate one-trial conditioning procedure, i.e. sucrose (CS)–KCl (US). In this cohort, 24 of the 50 snails met the criterion of good performers ($P<0.01$ *vs* pre-test, Student's *t*-test; Fig. 3A). All 50 snails were then given three extinction sessions (i.e. the CS was presented alone and was not paired with the US) at 10 min intervals after the one-trial conditioning procedure (i.e. 10 min, 20 min and 30 min after the conditioning procedure). We then subjected the snails to a memory test 1 h and 24 h later. As can be seen, memory was present when tested 1 h and 24 h later in the good performers ($P<0.01$ *vs* pre-test; Fig. 3A). That is, memory was present, and thus did not undergo extinction. Notice also that in the poor performers there was no significant change in the feeding response to the CS in any of the sessions following the one-trial conditioning procedure ($P>0.05$ for each comparison).

The second cohort of 50 snails received carrot juice rather than sucrose as the CS (Fig. 3B). This cohort was then given the appropriate one-trial conditioning procedure, i.e. carrot juice (CS)–KCl (US). The results were similar to those in the experiments where sucrose served as the CS. In this cohort, there were 21 snails that met the criterion for classification as

good performers, leaving the other 29 as poor performers. We again challenged these snails with the extinction protocol and found that extinction of CTA did not occur in the good performers. Therefore, we conclude that the LTM formed following the one-trial CTA procedure can be considered as a strong memory, because it is resistant to extinction.

Immediate cooling and blockade of long-term memory formation

A defining feature of LTM is that its formation (i.e. the consolidation process) following learning requires both altered gene activity and *de novo* protein synthesis (Davis and Squire, 1984; Milner et al., 1998; Kandel and Squire, 1999; McGaugh and Izquierdo, 2000; Kandel, 2001; Sangha et al., 2003a; Epstein et al., 2003; Epstein et al., 2004; Ribeiro et al., 2005; Azami et al., 2006; Wagatsuma et al., 2006). An advantage of using a one-trial conditioning procedure that results in LTM formation is that the consolidation process must begin at some time following the single pairing of the stimuli; providing a precise point in time to apply amnesiac agents. This fact may

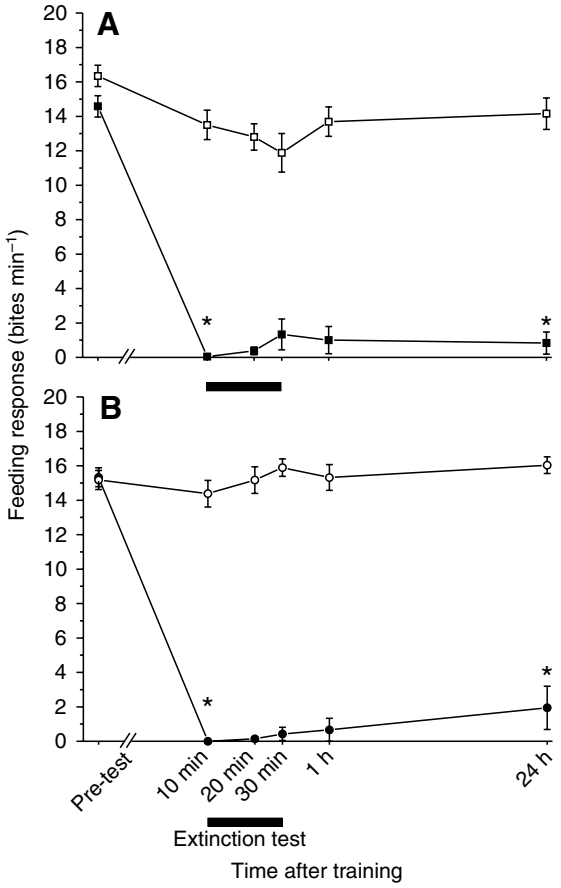


Fig. 3. Conditioned taste aversion (CTA) following the one-trial conditioning procedure is resistant to extinction. (A) The feeding response to the sucrose conditioned stimulus (CS) before conditioning, after conditioning and after three extinction sessions for good (open circles; *N*=24) and poor (solid circles; *N*=26) performers. (B) As in A, except that carrot juice was used as the CS. There were 21 good performers and 29 poor performers. Extinction training consisted of presentation of only the CS. The CS was presented three times with a 10-min interval after the conditioning procedure (bars). All values are means ± s.e.m. The *x* axes are in a logarithmic scale. The differences between the feeding response of the good performers and that of the poor performers in both cases were observed for at least 24 h at **P*<0.01 (Student's *t*-test), showing that the memory formed by the one-trial conditioning procedure is resistant to extinction.

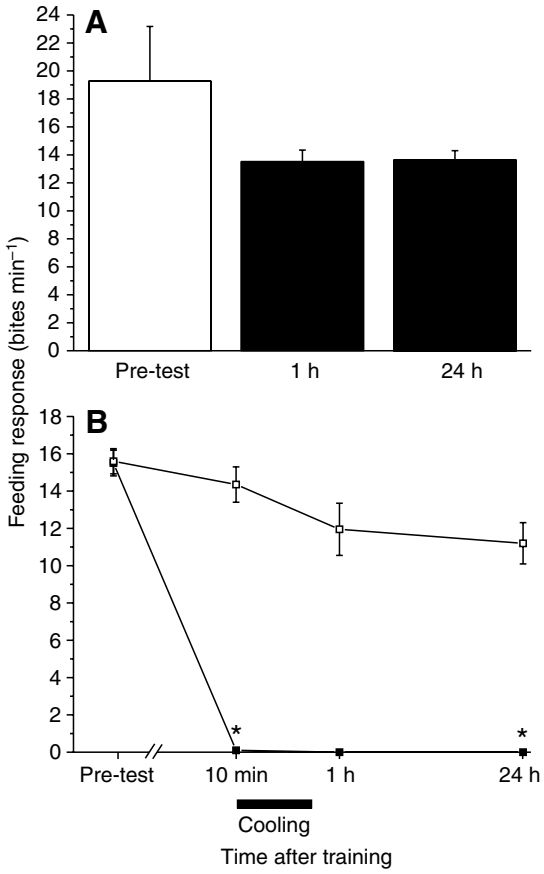


Fig. 4. Immediate cooling after the one-trial conditioning procedure blocks long-term memory (LTM) formation. (A) Snails (*N*=60) were subjected to the one-trial conditioning procedure and were then immediately (within 30 s) cooled to 4°C for 30 min. After cooling, snails were kept at room temperature and tested 1 h (*N*=30) and 24 h (*N*=30) later. There were no significant differences in the response to the conditioned stimulus (CS) following conditioning and the immediate cold block (one-way ANOVA). (B) Feeding response to the CS following the one-trial conditioning procedure after delayed cooling. Snails were cooled as in A except that the cooling was delayed until after the 10-min memory test. That is, rather than immediate cooling, cooling was delayed for 11 min. In this experiment we classified 20 snails as good performers (filled squares) and 10 (open squares) as poor performers. The bar below the graph indicates the cooling period. The *x* axis has a logarithmic scale. The differences between the feeding response of the good performers and that of the poor performers were observed for at least 24 h (**P*<0.01, Student's *t*-test). All values are means ± s.e.m. These data show that the necessary new protein synthesis required for LTM is initiated within 10 min of conditioning.

allow us to determine more precisely both when and how quickly LTM forms.

We first determined the feeding response to sucrose (the CS) in the pre-test session of another cohort (N=30) of snails randomly collected from the water tank. These snails were then subjected to the one-trial conditioning procedure. Immediately following the procedure (within 5 s), all 30 snails were placed into cooled (4°C) DW and were maintained at this temperature for 30 min. Following this 30-min cooling, all the snails were placed into room temperature (20°C) DW for at least 20 min before the post-test session. We then tested all 30 snails for memory 1 h and 24 h later (Fig. 4A). We found that in both the 1 h and 24 h post-test sessions, none of the snails (i.e. 0/30) met the criterion for good performers, and when we statistically compared the feeding response elicited by the CS in each of the post-test sessions (i.e. the 1 h and 24 h post-tests), it was not different from that in the pre-test session (P>0.05, one-way ANOVA; Fig. 4A). That is, memory was not observed. Note that in this experiment because LTM formation was blocked there were no good performers. All snails could be categorized as poor performers. Thus the immediate application of the cold block prevented LTM formation.

We next used another cohort of 30 snails randomly collected from the water tank. Again their feeding response to the sucrose CS was measured in the pre-test session. These snails were then subjected to the one-trial conditioning procedure, and their feeding response to the CS was determined 9 min and 30 s later (see Fig. 1A). Following this first memory test, the snails were placed in the cooled 4°C DW for 30 min (Fig. 4B). That is, we delayed placing the snails into the cooled 4°C DW for 10 min 30 s after the one-trial conditioning procedure. The snails were then maintained in the cooled 4°C DW for 30 min before being placed into DW at room temperature for at least 20 min. Then, a second post-test for memory was performed 1 h after the one-trial conditioning procedure and a third test was performed 24 h later after the procedure. In this second cohort of 30 snails we found that 20 of the 30 snails met the criterion of good performers in the first post-test session (P<0.01 *vs* pre-test, Student's t-test).

If we compare the first cohort of 30 snails with the second cohort of 30 snails, we see one big difference. There were no good performers in the first cohort. All 30 of the snails were poor performers – that is, none showed LTM. In the second cohort, on the other hand, 20 of the 30 snails were classified as good performers, and only 10 were poor performers. The only difference in the experimental procedure applied to the two cohorts was that the cooling was applied immediately after the single pairing of the CS–US in the first cohort, whereas it was delayed for 10 min in the second cohort. Thus we conclude that cooling immediately after conditioning blocks LTM formation. Because 40 of the 60 snails were classified as poor performers, meaning that they exhibited a robust feeding response to the CS in the post-test, we could conclude that 30 min cooling did not inhibit the ability of snails to feed. Taken together, these data show that: (1) the consolidation process for LTM occurs within 10 min following the one-trial conditioning procedure; and (2) 10 min cooling does not disrupt the LTM.

Inhibitory learning for conditioned taste aversion

The ability to learn and subsequently form LTM may be positively or negatively affected by previous experience. For example, in honeybees, Menzel and colleagues demonstrated that learning is impeded if false information is presented before correct information (Hammer and Menzel, 1995; Gerber et al., 1998). This is referred to as inhibitory learning. We hypothesized that such inhibitory learning would impair the acquisition and memory formation of CTA in *Lymnaea*.

We therefore used two further cohorts of snails (N=50 snails in each cohort; both randomly collected from the water tank) to determine if a backward-conditioning procedure prevents or impairs the formation of LTM following the CTA procedure (Fig. 1C). The first cohort of 50 snails received 10 presentations of DW paired with DW (i.e. the naive control procedure; see Materials and methods). Following this procedure, we then conditioned the snails using the one-trial conditioning procedure (i.e. a single sucrose–KCl pairing). We found that the presentation of the naive-control procedure before the one-trial conditioning procedure did not impair the formation of LTM, as the percentages of snails that met the criterion of a good performer when tested either 10 min or 7 days after the one-trial conditioning session were similar to the values shown in Figs 2 and 3 (Table 1). In other words, the paired presentation of DW to the snails did not alter their ability to make the association between sucrose and KCl.

We next challenged the second cohort of 50 snails to the inhibitory learning procedure (i.e. backward-conditioning procedure: 10-paired presentation of the US before the CS)

Table 1. *Backward-conditioning effects as inhibitory learning*

	N	Time after conditioning	Number of good performers	Number of poor performers
Control	50	10 min	21	29
		7 days	16	34
Backward conditioning	50	10 min	0	50
		7 days	0	46*

In the control, snails were trained by the naive procedure (10 presentations of distilled water) and then the one-trial conditioned taste aversion (CTA) procedure (sucrose and KCl). In the backward conditioning, snails were trained by the backward-conditioning procedure (10 pairings of KCl and sucrose) and then the one-trial CTA procedure (sucrose and KCl). *Four snails withdrew their body into the shell or died.

before the one-trial conditioning procedure (i.e. CS–US). Now none of the snails met the criterion of a good performer either 10 min or 7 days following the one-trial conditioning session (Table 1). That is, we routinely expect that approximately 40% of snails will form LTM following the one-trial conditioning procedure; but if we perform the backward conditioning procedure before the CTA procedure, none of the snails form LTM. We therefore concluded that the ability to form LTM following the CTA procedure is impaired or blocked by prior exposure of the snails to 'misinformation'; that is, the CS does not predict the subsequent application of the US.

Inhibitory learning and taste discrimination

We next asked whether the inhibitory effect of backward conditioning on LTM formation was context specific. That is, if we used sucrose as the CS and KCl as the US in the backward-conditioning procedure, would this misinformation block the acquisition of CTA when carrot juice rather than sucrose was used as the CS? We have previously found that snails discriminate among different tastes during the multi-trial CTA procedure (Sugai et al., 2006). To answer this question, we used another cohort of 50 snails that were randomly collected from the water tank. These snails received the backward-conditioning procedure as described above (10 trials of US–CS pairing) with sucrose as the CS. Following the backward-conditioning procedure, we subjected these snails to the one-trial conditioning procedure. However, carrot juice (CS2), not sucrose (CS1), was used as the CS in the one-trial conditioning session. We found that when the snails were challenged with carrot juice (CS2) as the CS in both the 10 min and 7 day post-test sessions, the numbers of snails in which memory was present were similar to the numbers in Figs 2 and 3. When tested 10 min after the one-trial conditioning procedure, 50% of snails (25 of 50 snails) were classified as good performers whereas 7 days later 24% of snails (11 of 45 snails) achieved this classification (Table 2). That is, backward conditioning with sucrose as the CS did not prevent memory formation when carrot juice was used as the CS in the one-trial conditioning procedure (Table 2). Thus, the ability of misinformation to inhibit the formation of new LTM is context specific.

At the same time that we performed the above experiment testing the context specificity of backward conditioning, we also repeated the experiment in which inhibitory learning (i.e. backward conditioning) blocked the establishment of memory with the one-trial conditioning procedure (Table 2). Thus a fourth cohort of 50 snails received 10 trials of backward conditioning (US–CS1 with KCl as the US and sucrose as the CS) and one-trial of CS1–US (sucrose–KCl). In this experiment only two snails met the criterion of good performers in the first memory test session (10 min) and only one snail met the criterion of a good performer in the 7 day memory test. Thus, we conclude that inhibitory learning makes it difficult for snails to undergo one-trial learning and form LTM. However, inhibitory learning in one context does not prevent LTM formation if another context is used as the CS in the one-trial conditioning procedure. Again, this underscores the importance of context in memory formation and recall.

Food deprivation and motivation

A possible explanation for the finding that only a minority of snails (i.e. the good performers) were able to form LTM following our one-trial CTA procedure is that the snails showing poor performance were not sufficiently motivated to acquire learning. Motivation plays an important role in learning (e.g. Frenois et al., 2005). Put another way, the good performers may have been more highly motivated than the poor performers, and thus learned and formed memories. To examine this possibility, we attempted to increase the motivation level of all snails by food deprivation. We deprived a cohort of snails (N=40) of food for 5 days and then subjected them to the one-trial CTA procedure (Fig. 1A and Fig. 5A).

In addition, we used a second cohort of snails deprived of food for 5 days (N=40) and subjected them to the 10-trial CTA procedure (Fig. 1B and Fig. 5B). Rather than enhancing memory formation, the 5-day food deprivations completely blocked memory formation in both cohorts. Thus, rather than increasing the ability of the snails to learn, the 5-day food deprivation had the opposite effect. Food-deprived snails were all classified as poor performers. Two possible explanations for these findings are: (1) the 5 day food deprivation made the snails sick and thus incapable of forming memory; (2) the long period of food deprivation caused excessive stress in the snails, which inhibited memory formation.

Discussion

In the present study, we began to address the question of why some snails are capable of associative learning and subsequently forming LTM following a one-trial CTA

Table 2. *Relation between backward-conditioning procedure and one-trial conditioned taste aversion conditioning*

	N	Time after conditioning	Number of good performers	Number of poor performers
(US+CS1)×10 and CS2+US	50	10 min	25	25
		7 days	11*	34
(US+CS1) and CS1+US×10	50	10 min	2	48
		7 days	1	44*

CS, conditioned stimulus; US, conditioned stimulus; CS1, sucrose; CS2, carrot juice.
*Five snails withdrew their body to the shell or died.

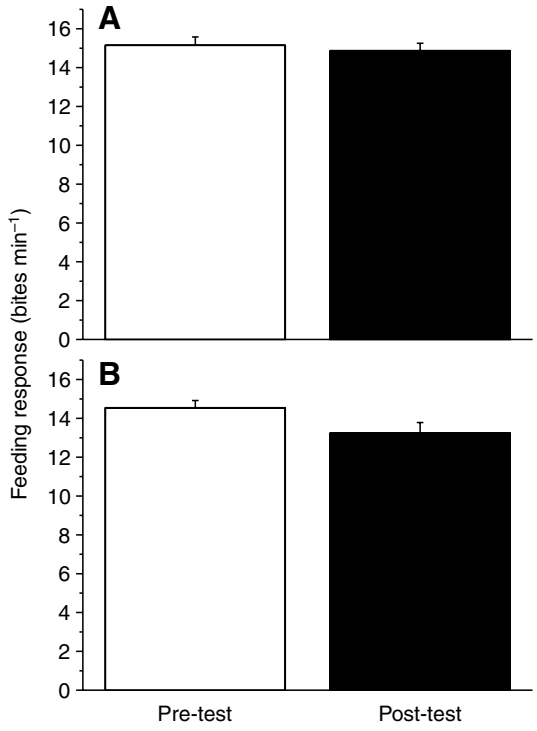


Fig. 5. Food deprivation for 5 days inhibits conditioned taste aversion (CTA) memory formation. (A) The response to the conditioned stimulus (CS) in the pre- and post-test sessions following the one-trial conditioning procedure in snails deprived of food for 5 days. Memory was not formed as indicated by there being no significant difference between the pre-test and post-test response to the CS. (B) As in A, except that these snails received 10 paired backward-conditioning (CS–US) presentations. Again, long-term memory (LTM) was not observed even with this more intense conditioning. In both A and B 40 snails were used. No significant differences in the feeding response were found in either case (Student's *t*-test). All data are the means ± s.e.m. (*N*=40 each). These results show that 5 days of food deprivation alters the internal state of the snail in such a manner that memory for CTA cannot be formed.

procedure whereas other similarly reared snails from the same stock do not have this capability. Before we can formulate a viable, testable hypothesis at the cellular and molecular levels (e.g. the presence of different ratios of two types of cyclic-AMP responsive element binding protein in key neurons for *Lymnaea* CTA) (Sadamoto et al., 2004a; Wagatsuma et al., 2005; Wagatsuma et al., 2006), we must first examine a number of the important parametric characteristics of this one-trial CTA learning (e.g. the formation, strength and modifiability of LTM).

Good versus poor performers

Our first finding was that approximately 40% of snails (the good performers) had the capability of making an association between a single paired presentation of an appetitive CS and an adversative US and then committing that new learned behavior to LTM. Namely, following the single pairing of the CS and US, the CS no longer elicited a feeding response in these snails 10 min (i.e. associative learning) and 1 week (i.e. LTM) following the conditioning (Fig. 2). We obtained similar results whether we used sucrose or carrot juice as the CS (Fig. 3).

Our initial hypothesis was that there would be a significant difference in how good and poor performers responded to the CS. However, when we examined the pre-conditioning response of snails to either the sucrose or carrot juice CS, no significant difference in the elicited feeding response to the CS could be observed between those snails that would later be classified, on the establishment or not of LTM, as good or poor performers. That is, we could not predict whether a snail would later be classified as a good or poor performer following the one-trial conditioning procedure based on the snail's response to the CS in the pre-test session. Because all snails showed a robust and similar feeding response to either CS in the pre-test session, we also concluded that the snails were healthy. Thus, those snails that ended up being classified as poor performers did not appear to be sick.

Food deprivation for 1 day does not alter the mortality or morbidity of snails; in fact it has been shown that 1 day of deprivation facilitates the ability to transport snails from Amsterdam to Sapporo or Calgary to Sapporo (i.e. decreases the number of snails dying in passage). In addition, none of the 1 day food-deprived snails appeared lethargic and all snails exhibited the typical exploratory behavior when placed in a new environment (i.e. the Petri dish). We therefore could not conclude that the ability or inability to learn and form memory was the result of a difference in responding to the CS or in the health status of the snails.

Another possible explanation for the difference in ability to learn and form LTM seen in our present study is that it was related to the use of different populations of snails. However, we believe that we can discount this possibility, because all snails used in our present study were derived from the original snail colony in Amsterdam, and they were all of the same size and age. Further, Lukowiak et al. reported learning and memory data for over 3000 *Lymnaea* given operant conditioning of aerial respiratory behavior over a time course of 10 years by various researchers in a single laboratory (Lukowiak et al., 2003). There were no differences in the learning or memory curves obtained over that time period. Thus, we have been dealing with a stable, relatively homogenous population of *Lymnaea* that were originally derived from Vrije Universiteit Amsterdam. However, it also has to be pointed out that in the study by Lukowiak et al. approximately 20% of snails were classified as having poor ability to form LTM following conditioning (Lukowiak et al., 2003). Thus, within a large population of subjects a certain subset of snails, following conditioning, fails to form LTM. Why these differences occur in seemingly identical subjects is not understood.

We also thought it was possible that there might have been a difference between the good and poor performers as regards their sensitivity to the aversive US (i.e. the KCl). However, all snails whether they would later be classified as good or poor

performers stopped feeding and locomoting when the aversive US was presented. Nor did the snails that would later be classified as poor performers begin to locomote any sooner or later than the snails that came to be classified as good performers (data not shown). Thus, at this point we have no easily discernable behavioral phenotypic difference that would allow an investigator to predict whether a snail would learn and form LTM after a one-trial CTA procedure.

It would seem a logical next step to obtain offspring from good and poor performers and then determine if there was a genetic basis to the inability to form LTM. However, this was not possible in the present series, because we used adult snails, and thus it was not possible to be certain of the paternity or maternity of the individual egg clutches. *Lymnaea* are hermaphrodites that are capable of storing sperm for long periods of time before the eggs are fertilized and then laid (ter Maat, 1992). Thus, even if a poor performer was placed together with another poor performer and eggs were seen later on, one could not be certain that a good performer had not deposited sperm before any test was performed. One way around this issue would be to isolate snails as soon as they were only a few weeks old, before they were sexually active, then determine if they were good or poor performers and make the appropriate pairings. Whether or not being reared in isolation would result in any abnormalities of behavior is uncertain. *Lymnaea* are also capable of self-fertilization (termed 'selfing') (e.g. Coutellec and Lagadic, 2006), which also could complicate the subsequent analysis. In any case we have not yet performed these experiments.

In a somewhat analogous way, it appears to be difficult to discern, before conditioning, a phenotypic behavioral difference in many of the *Drosophila* mutants that exhibit marked differences in their ability to learn or remember (e.g. Dubnau and Tully, 1998). However, this does not mean that we may not yet discover distinguishable differences in snails that would allow us to predict whether they will learn and form LTM before conditioning. For example, in studies using siphon, mantle, gill and abdominal ganglion preparations of *Aplysia*, the behavioral state of the animals before dissection predicted how the gill would respond to tactile stimulation of the siphon (Lukowiak and Freedman, 1983; Lukowiak, 1987). Whether the intact *Aplysia* was food-satiated or had been engaged in sexual activity just before dissection had a significant and predictable impact on both the amplitude of the reflex evoked by the standard tactile stimulus applied to the siphon or gill and the rate of habituation that occurred with repeated presentations of the tactile stimulus. Further, in previous reports on *Hermissenda* preparations, both good and poor performers have been observed (Alkon et al., 1990; Ito et al., 1994). As we found here, it was not possible to predict beforehand which animals would learn and remember before conditioning.

The previous comparison between good and poor performers in *Lymnaea* that were conditioned by eight pairings of CS and US showed that the inhibitory postsynaptic potentials (IPSPs) recorded in the N1 medial (N1M) cells in the good performers were significantly larger ($P<0.05$) than those of backward-conditioning and naive controls after the depolarization of the cerebral giant cells. However, the IPSPs of the poor performers were not significantly different from those of the controls or the good performers (Kojima et al., 1997). Therefore it is probable that IPSPs in the N1M cells in the brains of poor performers are not easily formed by the CTA procedure.

Cooling effects on formation of long-term memory

An advantage of a one-trial conditioning procedure is that the initiation of the consolidation process can be better pinpointed than when multiple conditioning sessions or single conditioning sessions of longer duration (e.g. tens of minutes) are used. As we have shown in the present study, consolidation begins within 10 min after the pairing of the CS-US. We arrived at this conclusion based on the fact that we could block LTM formation if we immediately cooled snails to 4°C and maintained them at this temperature for 30 min (Fig. 4A). If we delayed the 30 min cooling by only 10 min, LTM was not blocked (Fig. 4B). We have not, as yet, attempted to determine the shortest duration of cooling that is sufficient to inhibit memory formation. These experiments are planned and the results may give us an indication of the duration of consolidation process.

It has been hypothesized that cooling snails to 4°C blocks the altered gene activity and *de novo* protein synthesis processes that are required for LTM formation (e.g. Sangha et al., 2003b). Delaying the imposition of the cooling for 10 min must therefore allow the molecular processes that cause memory formation to reach a state in which they become invulnerable to the disruptive effects of cooling on memory. Thus, if cooling is to be effective in blocking LTM formation it must be immediately imposed following the conditioning session. A similar conclusion was arrived at in studies also performed in *Lymnaea* using two different experimental protocols. Following a one-trial appetitive conditioning procedure, amnesiac agents injected 10 min to 1 h after conditioning were effective in blocking LTM formation (Fulton et al., 2005), and cooling to block LTM in the aerial respiratory operant conditioning was only effective in blocking LTM if applied within 10 min following the conditioning procedure (Sangha et al., 2003b).

These data are similar to those that show that the application of other amnesiac agents (e.g. anisomycin or actinomycin D) after conditioning has occurred is ineffective in blocking memory formation (e.g. Matsuo et al., 2002; Yasui et al., 2004). However, the advantage that cooling has over other such amnesiac agents is that it can be applied quickly, it is relatively harmless, and it can be reversed quickly. Knowing that the molecular processes that underlie memory formation occur within 10 min after the one-trial conditioning session may allow us to differentiate between causal and correlative molecular events in key neurons that are necessary for behavioral memory (Azami et al., 2006; Wagatsuma et al., 2006). That is, if a change in a molecular marker does not occur within 10 min of the termination of the one-trial conditioning

procedure then it is probably not an early, causal, necessary process for memory formation.

Backward conditioning as inhibitory learning

Whereas the one-trial conditioning procedure used in our present study is a potent one, in that it causes LTM formation persisting for at least 1 week in a significant number of snails; its potency can be degraded (Fig. 2). This erosion of potency can be accomplished by altering the state of the snails before the presentation of the single CS-US pairing. For example, if the snails are first given inhibitory learning (backward conditioning), the one-trial conditioning procedure does not cause LTM formation in any of the snails (Table 1). In a backward-conditioning procedure, we hypothesize that snails learn and remember that the CS does not signal the arrival of the US; thus this negative association has to be overcome before the snails are capable of learning that the CS will now signal the arrival of the US (e.g. Mpitsos and Collins, 1975).

Moreover, it was not just the presentation of extra stimuli or handling of the snails prior to the one-trial conditioning that interfered with the snails' ability to form LTM, as snails exposed to 'naive-conditioning' were still capable of forming LTM following the one-trial conditioning procedure. Additionally, for backward conditioning to be effective in blocking LTM formation following the one-trial conditioning, the inhibitory learning had to occur in the same context as the subsequent conditioning. Thus, if backward conditioning was performed using sucrose as the CS and then one-trial conditioning was performed using carrot juice as the CS, LTM formation occurred in the same percentage of snails as in the trial in which backward conditioning was omitted. These data again emphasize how important context is in memory formation and recall (Parvez et al., 2005; Parvez et al., 2006).

Failure of extinction

Not only does the CTA memory persist for at least 7 days following the one-trial conditioning session, but this memory is also resistant to the extinction process (Fig. 3). Extinction has previously been demonstrated in *Lymnaea* (Sangha et al., 2003a) following operant conditioning of aerial respiratory behavior. Extinction is not the erasure of the old memory, but rather is new learning and memory that temporarily occludes the old memory (Berman and Dudai, 2001). The memory for extinction typically does not persist very long, such that the old memory re-appears. This issue has been known since the time of Pavlov and has been termed spontaneous recovery (Mackintosh, 1974).

If a memory is resistant to extinction conditioning, as is the memory for CTA, then this is an indication of how strong the memory is. That is, strong memories are difficult to extinguish (Bouton, 1993; Myers and Davis, 2002). Whether with more extinction conditioning sessions the memory for CTA would have become occluded remains to be determined. Thus, LTM in *Lymnaea* following a single pairing of the CS and US (i.e. our one-trial CTA procedure) is an extremely robust type of learning and memory, sharing many of the attributes of the "Garcia effect" of mammals (Garcia et al., 1985).

Motivation on conditioned taste aversion

In an attempt to increase the percentage of snails that can successfully acquire and form LTM following the one-trial CTA procedure, we attempted to increase the motivational state of the snails by depriving them of food for 5 days rather than 1 day. As has previously been demonstrated for appetitive one-trial conditioning (Alexander et al., 1984; Straub et al., 2004), longer periods of food deprivation were necessary to increase the success rate of the conditioning in producing LTM formation. In those experiments, food deprivation for 4–5 days enhanced the ability of snails to learn and form memory. However, in the present case, the opposite occurred. That is, depriving snails of food for 5 days resulted in a change in the motivational state, the net effect of which was that snails no longer learned or formed memory.

What is the reason for these disparate results? Clearly food deprivation for 5 days alters the motivational state of the snails. Depending on the specific conditioning procedure used, such food deprivation may enhance or retard the ability to learn and form memory. In the appetitive conditioning experiments, food deprivation makes it more likely that the snails will associate a neutral CS with food; we believe this is because hungry snails are more likely to pay attention and form memory for a stimulus that signals the availability of food. On the other hand, we hypothesize that when snails are in a food-deprived state and are presented with food as a CS, they are so intent on feeding that they choose not to make an association between the food stimulus (CS) and the US that causes cessation of feeding.

A similar finding had previously been shown in *Lymnaea*. Haney and Lukowiak demonstrated that aerial respiratory behavior could be operantly conditioned using a carrot smell, but only when snails were not food-deprived (Haney and Lukowiak, 2001). When snails were deprived of food, they did not learn or form memory in the presence of a carrot smell. However, the same food-deprived snails learned and formed memory when the carrot smell was not present, showing that it was not food-deprivation *per se* that inhibited learning and memory. Snails that are deprived of food for 5 days and then smell food are more likely to pay attention to stimuli that signal the presence of food rather than to stimuli that do not result in satisfying the drive to attain food. It is also possible that snails deprived of food for 5 days are too stressed to learn and form memory when a CS such as sucrose or carrot juice is paired with an aversive US. Thus, the data obtained in the present study and previous studies are consistent with the hypothesis that food deprivation by itself is not causal to blocking the formation of memory. Rather, the alteration in the internal state of the snail resulting from food-deprivation may block learning and/or memory formation when stimuli used as the US or reinforcing stimuli in operant conditioning do not result in drive reduction (i.e. satisfying the hunger). Therefore, the data obtained in *Lymnaea* thus far are inconsistent with the notion

that food deprivation results in unhealthy snails that do not have the capacity to learn and form memory.

Our previously published work tended to focus on group data and on experimental procedures optimized to produce consistent learning and LTM formation (Kojima et al., 1996; Wagatsuma et al., 2004). Thus, individual differences in learning and memory capabilities between subjects were not a major focus. In the present study, we propose that such differences may provide clues to a mechanistic explanation of why some subjects learn and remember more easily than others. It is clear that we have now developed a one-trial CTA procedure that results in a consistent percentage of snails forming LTM. It is also clear that if snails have memory at the 10 min interval following the one-trial conditioning then they will likely have LTM when tested 7 days later. We are thus at a stage where we can begin to examine the neuronal and molecular differences between good and poor performers. Having this knowledge will allow us to better elucidate the causal mechanisms of memory formation and possibly to answer the question of why some individuals are better at learning and forming memory for specific tasks.

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References

Alexander, J., Audesirk, T. E. and Audesirk, G. J. (1984). One-trial reward learning in the snail *Lymnaea stagnalis*. *J. Neurobiol.* **15**, 67-72.

Alkon, D. L., Ikeno, H., Dworkin, J., McPhie, D. L., Olds, J. L., Lederhendler, I., Matzel, L., Schreurs, B. G., Kuzirian, A., Collin, C. et al. (1990). Contraction of neuronal branching volume: an anatomic correlate of Pavlovian conditioning. *Proc. Natl. Acad. Sci. USA* **87**, 1611-1614.

Azami, S., Wagatsuma, A., Sadamoto, H., Hatakeyama, D., Usami, T., Fujie, M., Koyanagi, R., Azumi, K., Fujito, Y., Lukowiak, K. et al. (2006). Altered gene activity correlated with long-term memory formation of conditioned taste aversion in *Lymnaea*. *J. Neurosci. Res.* **84**, 1610-1620.

Benjamin, P. R., Staras, K. and Kemenes, G. (2000). A systems approach to the cellular analysis of associative learning in the pond snail *Lymnaea*. *Learn. Mem.* **7**, 124-131.

Berman, D. E. and Dudai, Y. (2001). Memory extinction, learning anew, and learning the new: dissociations in the molecular machinery of learning in cortex. *Science* **291**, 2417-2419.

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychol. Bull.* **114**, 80-99.

Coutellec, M. A. and Lagadic, L. (2006). Effects of self-fertilization, environmental stress and exposure to xenobiotics on fitness-related traits of the freshwater snail *Lymnaea stagnalis*. *Ecotoxicology* **15**, 199-213.

Davis, H. P. and Squire, L. R. (1984). Protein synthesis and memory: a review. *Psychol. Bull.* **96**, 518-559.

Dubnau, J. and Tully, T. (1998). Gene discovery in *Drosophila*: new insights for learning and memory. *Annu. Rev. Neurosci.* **21**, 407-444.

Epstein, H. T., Child, F. M., Kuzirian, A. M. and Alkon, D. L. (2003). Time windows for effects of protein synthesis inhibitors on Pavlovian conditioning in *Hermissenda*: behavioral aspects. *Neurobiol. Learn. Mem.* **79**, 127-131.

Epstein, H. T., Kuzirian, A. M., Child, F. M. and Alkon, D. L. (2004). Two different biological configurations for long-term memory. *Neurobiol. Learn. Mem.* **81**, 12-18.

Frenois, F., Le Moine, C. and Cador, M. (2005). The motivational component of withdrawal in opiate addiction: role of associative learning and aversive memory in opiate addiction from a behavioral, anatomical and functional perspective. *Rev. Neurosci.* **16**, 255-276.

Fulton, D., Kemenes, I., Andrew, R. J. and Benjamin, P. R. (2005). A single time-window for protein synthesis-dependent long-term memory formation after one-trial appetitive conditioning. *Eur. J. Neurosci.* **21**, 1347-1358.

Garcia, J., Kimeldorf, D. J. and Koelling, R. A. (1955). Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science* **122**, 157-158.

Garcia, J., Hankins, W. G. and Rusiniak, K. W. (1974). Behavioral regulation of the melieu interne in man and rat. *Science* **185**, 824-831.

Garcia, J., Lasiter, P. S., Bermúdez-Rattoni, F. and Deems, D. A. (1985). A general theory of aversion learning. *Ann. NY Acad. Sci.* **443**, 8-21.

Gerber, B., Wustenberg, D., Schutz, A. and Menzel, R. (1998). Temporal determinants of olfactory long-term retention in honeybee classical conditioning: nonmonotonous effects of the conditioning trial interval. *Neurobiol. Learn. Mem.* **69**, 71-78.

Hammer, M. and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci.* **15**, 1617-1630.

Haney, J. and Lukowiak, K. (2001). Context learning and the effect of context on memory retrieval in *Lymnaea*. *Learn. Mem.* **8**, 35-43.

Hatakeyama, D., Sadamoto, H. and Ito, E. (2004a). Real-time quantitative RT-PCR method for estimation of mRNA level of CCAAT/enhancer binding protein in the central nervous system of *Lymnaea stagnalis*. *Acta Biol. Hung.* **55**, 157-161.

Hatakeyama, D., Fujito, Y., Sakakibara, M. and Ito, E. (2004b). Expression and distribution of transcription factor CCAAT/enhancer-binding protein in the central nervous system of *Lymnaea stagnalis*. *Cell Tissue Res.* **318**, 631-641.

Hatakeyama, D., Sadamoto, H., Watanabe, T., Wagatsuma, A., Kobayashi, S., Fujito, Y., Yamashita, M., Sakakibara, M., Kemenes, G. and Ito, E. (2006). Requirement of new protein synthesis of a transcription factor for memory consolidation: paradoxical changes in mRNA and protein levels of C/EBP. *J. Mol. Biol.* **356**, 569-577.

Ito, E., Oka, K., Collin, C., Schreurs, B. G., Sakakibara, M. and Alkon, D. L. (1994). Intracellular calcium signals are enhanced for days after Pavlovian conditioning. *J. Neurochem.* **62**, 1337-1344.

Ito, E., Kobayashi, S., Kojima, S., Sadamoto, H. and Hatakeyama, D. (1999). Associative learning in the pond snail, *Lymnaea stagnalis*. *Zool. Sci.* **16**, 711-723.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**, 1030-1038.

Kandel, E. R. and Squire, L. R. (2000). Neuroscience: breaking down scientific barriers to the study of brain and mind. *Science* **290**, 1113-1120.

Kawai, R., Sunada, H., Horikoshi, T. and Sakakibara, M. (2004). Conditioned taste aversion with sucrose and tactile stimuli in the pond snail *Lymnaea stagnalis*. *Neurobiol. Learn. Mem.* **82**, 164-168.

Kemenes, G., Kemenes, I., Michel, M., Papp, A. and Müller, U. (2006). Phase-dependent molecular requirements for memory reconsolidation: differential roles for protein synthesis and protein kinase A activity. *J. Neurosci.* **26**, 6298-6302.

Kemenes, I., Kemenes, G., Andrew, R. J., Benjamin, P. R. and O'Shea, M. (2002). Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *J. Neurosci.* **22**, 1414-1425.

Kemenes, I., Straub, V. A., Nikitin, E. S., Staras, K., O'Shea, M., Kemenes, G. and Benjamin, P. R. (2006). Role of delayed nonsynaptic neuronal plasticity in long-term associative memory. *Curr. Biol.* **16**, 1269-1279.

Kobayashi, S., Kojima, S., Yamanaka, M., Sadamoto, H., Nakamura, H., Fujito, Y., Kawai, R., Sakakibara, M. and Ito, E. (1998). Operant conditioning of escape behavior in the pond snail, *Lymnaea stagnalis*. *Zool. Sci.* **15**, 683-690.

Kojima, S., Yamanaka, M., Fujito, Y. and Ito, E. (1996). Differential neuroethological effects of aversive and appetitive reinforcing stimuli on associative learning in *Lymnaea stagnalis*. *Zool. Sci.* **13**, 803-812.

Kojima, S., Nakamura, H., Nagayama, S., Fujito, Y. and Ito, E. (1997). Enhancement of an inhibitory input to the feeding central pattern generator in *Lymnaea stagnalis* during conditioned taste-aversion learning. *Neurosci. Lett.* **230**, 179-182.

Kojima, S., Ogawa, H., Kouuchi, T., Nidaira, T., Hosono, T. and Ito, E. (2000). Neuron-independent Ca^{2+} signaling in glial cells of snail's brain. *Neuroscience* **100**, 893-900.

Kojima, S., Hosono, T., Fujito, Y. and Ito, E. (2001). Optical detection of neuromodulatory effects of conditioned taste aversion in the pond snail *Lymnaea stagnalis*. *J. Neurobiol.* **49**, 118-128.

Lowe, M. R. and Spencer, G. E. (2006). Perturbation of the activity of a single

identified neuron affects long-term memory formation in a molluscan semi-intact preparation. *J. Exp. Biol.* **209**, 711-721.

Lukowiak, K. (1987). A blood-borne factor from food-satiated *Aplysia* suppresses the gill withdrawal reflex in in vitro preparations from unsatiated animals. *Neurosci. Lett.* **77**, 205-208.

Lukowiak, K. and Freedman, L. (1983). The gill withdrawal reflex is suppressed in sexually active *Aplysia*. *Can. J. Physiol. Pharmacol.* **61**, 743-748.

Lukowiak, K., Sangha, S., McComb, C., Varshney, N., Rosenegger, D., Sadamoto, H. and Scheibenstock, A. (2003). Associative learning and memory in *Lymnaea stagnalis*: how well do they remember? *J. Exp. Biol.* **206**, 2097-2103.

Mackintosh, N. J. (ed.) (1974). Extinction. In *The Physiology of Animal Learning*, pp. 405-483. London: Academic Press.

Margulies, C., Tully, T. and Dubnau, J. (2005). Deconstructing memory in *Drosophila*. *Curr. Biol.* **15**, R700-R713.

Masugi, M., Yokoi, M., Shigemoto, R., Muguruma, K., Watanabe, Y., Sansig, G., van der Putten, H. and Nakanishi, S. (1999). Metabotropic glutamate receptor subtype 7 ablation causes deficit in fear response and conditioned taste aversion. *J. Neurosci.* **19**, 955-963.

Matsuo, R., Hitomi, T., Watanabe, S. and Kirino, Y. (2002). Delayed-onset amnesia caused by protein synthesis inhibition in odor-taste associative memory of the terrestrial slug *Limax valentianus*. *Neurosci. Lett.* **334**, 201-205.

McGaugh, J. L. and Izquierdo, I. (2000). The contribution of pharmacology to research on the mechanisms of memory formation. *Trends Pharmacol. Sci.* **21**, 208-210.

Milner, B., Squire, L. R. and Kandel, E. R. (1998). Cognitive neuroscience and the study of memory. *Neuron* **20**, 445-468.

Mpitsos, G. J. and Collins, S. D. (1975). Learning: rapid aversive conditioning in the gastropod mollusk *Pleurobranchaea*. *Science* **188**, 954-957.

Myers, K. M. and Davis, M. (2002). Behavioral and neural analysis of extinction. *Neuron* **36**, 567-584.

Parvez, K., Stewart, O., Sangha, S. and Lukowiak, K. (2005). Boosting intermediate-term into long-term memory. *J. Exp. Biol.* **208**, 1525-1536.

Parvez, K., Moisseev, V. and Lukowiak, K. (2006). A context-specific single contingent-reinforcing stimulus boosts intermediate-term memory into long-term memory. *Eur. J. Neurosci.* **24**, 606-616.

Rankin, C. H. (2005). Nematode memory: now, where was I? *Curr. Biol*. **15**, R374-R375.

Ribeiro, M. J., Schofield, M. G., Kemenes, I., O'Shea, M., Kemenes, G. and Benjamin, P. R. (2005). Activation of MAPK is necessary for long-term memory consolidation following food-reward conditioning. *Learn. Mem.* **12**, 538-545.

Rosenegger, D., Roth, S. and Lukowiak, K. (2004). Learning and memory in *Lymnaea* are negatively altered by acute low-level concentrations of hydrogen sulphide. *J. Exp. Biol.* **207**, 2621-2630.

Sadamoto, H., Yamanaka, M., Hatakeyama, D., Nakamura, H., Kojima, S., Yamashita, M. and Ito, E. (2000). Developmental study of anatomical substrate for conditioned taste aversion in *Lymnaea stagnalis*. *Zool. Sci.* **17**, 141-148.

Sadamoto, H., Sato, H., Kobayashi, S., Murakami, J., Aonuma, H., Ando, H., Fujito, Y., Hamano, K., Awaji, M., Lukowiak, K. et al. (2004a). CREB in the pond snail *Lymnaea stagnalis*. cloning, gene expression and function in identifiable neurons of the central nervous system. *J. Neurobiol.* **58**, 455-466.

Sadamoto, H., Azami, S. and Ito, E. (2004b). The expression pattern of CREB genes in the central nervous system of the pond snail *Lymnaea stagnalis*. *Acta Biol. Hung.* **55**, 163-166.

Sakakibara, M. (2006). Comparative study of visuo-vestibular conditioning in *Lymnaea stagnalis*. *Biol. Bull*. **210**, 298-307.

Sakakibara, M., Aritaka, T., Iizuka, A., Suzuki, H., Horikoshi, T. and Lukowiak, K. (2005). Electrophysiological responses to light of neurons in the eye and statocyst of *Lymnaea stagnalis*. *J. Neurophysiol.* **93**, 493-507.

Sangha, S., Scheibenstock, A., Morrow, R. and Lukowiak, K. (2003a). Extinction requires new RNA and protein synthesis and the soma of the cell right pedal dorsal 1 in *Lymnaea stagnalis. J. Neurosci.* **23**, 9842-9851.

Sangha, S., Morrow, R., Smyth, K., Cooke, R. and Lukowiak, K. (2003b). Cooling blocks ITM and LTM formation and preserves memory. *Neurobiol. Learn. Mem.* **80**, 130-139.

Spencer, G. E., Syed, N. I. and Lukowiak, K. (1999). Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnaea stagnalis*. *J. Neurosci.* **19**, 1836-1843.

Straub, V. A., Styles, B. J., Ireland, J. S., O'Shea, M. and Benjamin, P. R. (2004). Central localization of plasticity involved in appetitive conditioning in *Lymnaea*. *Learn. Mem.* **11**, 787-793.

Sugai, R., Shiga, H., Azami, S., Watanabe, T., Sadamoto, H., Fujito, Y., Lukowiak, K. and Ito, E. (2006). Taste discrimination in conditioned taste aversion of the pond snail *Lymnaea stagnalis*. *J. Exp. Biol.* **209**, 826-833.

ter Maat, A. (1992). Egg laying in the hermaphrodite pond snail *Lymnaea stagnalis. Prog. Brain Res.* **92**, 345-360.

Waddell, S. and Quinn, W. G. (2001). Flies, genes, and learning. *Annu. Rev. Neurosci.* **24**, 1283-1309.

Wagatsuma, A., Sugai, R., Chono, K., Azami, S., Hatakeyama, D., Sadamoto, H. and Ito, E. (2004). The early snail acquires the learning. Comparison of scores for conditioned taste aversion between morning and afternoon. *Acta Biol. Hung.* **55**, 149-155.

Wagatsuma, A., Sadamoto, H., Kitahashi, T., Lukowiak, K., Urano, A. and Ito, E. (2005). Determination of the exact copy numbers of particular mRNAs in a single cell by quantitative real-time RT-PCR. *J. Exp. Biol.* **208**, 2389-2398.

Wagatsuma, A., Azami, S., Sakura, M., Hatakeyama, D., Aonuma, H. and Ito, E. (2006). De novo synthesis of CREB in a presynaptic neuron is required for synaptic enhancement involved in memory consolidation. *J. Neurosci. Res.* **84**, 954-960.

Yasui, K., Matsuo, R. and Kirino, Y. (2004). Onset of amnesia is delayed with a decrease in inhibition of protein synthesis during odor-taste associative learning in the terrestrial slug *Limax valentianus*. *Zool. Sci.* **21**, 1163-1166.