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Boosting intermediate-term into long-term memory

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Summary

Aerial respiration in the pond snail *Lymnaea stagnalis* can be operantly conditioned. Depending on the specific training procedure used (i.e. a 0.5 h *vs* a 1.0 h interval between training sessions) either intermediate (ITM) or long-term memory (LTM) is formed. ITM, which persists for 2–3 h, is dependent only on *de novo* protein synthesis, whilst LTM persists for up to 4 weeks and is dependent on both transcription and *de novo* protein synthesis. We found that although the behavioural phenotype of ITM was not apparent 24 h after the last training session, a residual memory trace was present that serves as a foundation upon which a subsequent ITM-training-procedure builds on to form LTM (i.e. a 'changed memory'). This residual memory trace could be perturbed by cooling, the behavioural process of context-specific extinction and by increasing the interval between the training procedures. Furthermore in preparations where the somata of RPeD1 (one of three interneurons in the central pattern generator required for aerial respiratory behavior) had been ablated before training, LTM could not be observed following a second bout of ITM-training. These data support the concept that a molecular memory trace is established as a consequence of ITM-training, which serves as a 'permissive substrate', when the ITM memory is made active, sufficient to permit the necessary transcription and translation processes that are causal for LTM formation.

Key words: *Lymnaea stagnalis*, learning, operant conditioning, snail, behaviour, memory trace.

Introduction

Learning and memory are distinct but related processes, each with its own underlying neuronal and molecular mechanisms (Dudai, 2002a). The duration of memory depends on many factors, including the specific training procedure used (e.g. spaced *vs* massed learning), and long-lasting memory in *Lymnaea* can be categorized into intermediate-term memory (ITM; lasting 3 h) or long-term memory (LTM; lasting >6 h; Lukowiak et al., 2000). ITM requires new protein synthesis (i.e. translation of pre-existing mRNA) while LTM requires both transcription and translation (Rosenzweig, 1993; Sangha et al., 2003a; Sutton et al., 2002). Following learning there is a consolidation period (for both ITM and LTM), during which time the memory moves from a labile to a stable state (Nader, 2003; Walker et al., 2003). In this interlude, memory is perturbable by procedures, which obstruct the transcription and/or translation processes.

Lymnaea are bimodal breathers, therefore it is possible to modulate one of its respiratory behaviours (i.e. aerial respiration) while leaving the other (cutaneous) unaffected. We use a non-declarative, operant conditioning paradigm to decrease aerial respiratory behaviour (Lukowiak et al., 1996) and since the snails can still breathe cutaneously our procedure is not harmful. A three-neuron central pattern generator (CPG), whose sufficiency and necessity have been demonstrated, drives aerial respiratory behaviour (Syed et al., 1990, 1992). Since non-declarative memories are stored within the same network that mediates the behaviour (Dudai, 2002a), the changes induced by operant conditioning are stored within the respiratory CPG in *Lymnaea* (Spencer et al., 1999, 2002). In fact the molecular processes necessary for consolidation, reconsolidation (i.e. restabilization of the memory after it has been made active) and extinction of LTM occur within RPeD1 (Scheibenstock et al., 2002; Sangha et al., 2003b,c).

Previously we showed (Smyth et al., 2002) that although the behavioural phenotype of ITM was not apparent 5 h after training, there was enhancement of LTM persistence with subsequent LTM-training. We now extend these findings and show that ITM leaves behind a residual molecular memory trace, on which a second bout of ITM-training builds to cause the formation of LTM. We call this phenomenon 'memory boosting'. This 'boosting' of ITM to LTM occurs even if the behavioural manifestation of the memory is not apparent. However, this 'memory boosting' is (1) impeded by blocking new protein synthesis, (2) interfered with by behavioural extinction training, and (3) requires the presence of RPeD1's somata. These findings are all consistent with (1) the hypothesis that ITM and LTM formation occur in series (Ghirardi et al., 1995; Riedel, 1999; Zhao et al., 1995), and (2)

an emerging view that memory exists in either a labile or a stable state (Nader, 2003). Thus, when a memory is retrieved (i.e. activated) it re-enters the labile state and must go through a 'reconsolidation' phase in order for it to become stable and persist in the brain.

Materials and methods

Animals

Lymnaea stagnalis L., originally derived from stocks obtained from *Vrije Universeit* (Amsterdam), were bred and raised in the snail facility at the University of Calgary. Adult snails (shell length 23–26 mm) were maintained at room temperature (23°C) and had continuous access to lettuce in their home eumoxic (i.e. normal levels of O_2; 6 ml O_2 l^{-1}) aquaria.

Operant conditioning procedure

Individually labeled snails were placed in a 1 l beaker containing 500 ml of room temperature (19–20°C) hypoxic (<0.1 ml O_2 l^{-1}) water. The water was made hypoxic by bubbling N_2 through it 20 min prior to and during training and testing. Hypoxia dramatically increases aerial respiratory behaviour (Lukowiak et al., 1996; Rosenegger et al., 2004). Animals were first given a 10 min acclimatization period, during which they could freely perform aerial respiration. The onset of operant conditioning training was initiated by gently pushing the snails beneath the water surface. During the operant conditioning training session, every time a snail opened its pneumostome to perform aerial respiration, a sharpened wooden applicator (0.25 mm diameter) was used to 'poke' the pneumostome area to cause the animal to close the pneumostome. Withdrawal of the snail into its shell typically did not occur and most snails remained at the surface of the water. The gentle poke did not cause rotation of the snail and thus a statocyst-dependent reflex was probably not elicited. The time when every animal attempted to open its pneumostome was recorded. In between sessions, animals were kept in eumoxic pondwater and freely performed aerial respiration *ad libitum*. During the administration of a memory test (MT), animals were subjected to the application of tactile stimuli, as in operant conditioning training sessions.

We also utilized a 'change of context' testing procedure. To create the 'different context', N_2 was first bubbled through a 750 ml Erlenmeyer flask containing chopped carrots and water before being bubbled into the training beaker (Haney and Lukowiak, 2001). When sensing the presence of carrot odor, the animals perceive this as a different context and respond as if they have not received training; i.e. there is an increase in the number of pneumostome openings. The term 'change of context test' means that snails were tested in the context that they were *not* trained in. The term 'standard context' refers to the training procedure when nitrogen is bubbled directly into the beaker containing the snails; the term 'carrot-context' refers to the training procedure where nitrogen is first bubbled through the chopped carrots before it reaches the beaker containing the snails.

ITM and LTM training procedure

The ITM-training protocol consisted of two 30 min operant conditioning training sessions in hypoxic pondwater (TS) separated by a 30 min rest interval in eumoxic pondwater (Lukowiak et al., 2000). The LTM training procedure, on the other hand, consisted of two 30-minute operant conditioning training sessions separated by a 1 h rest interval (Lukowiak et al., 2000. A MT was presented to the snails 3 or 24 h after the last training session. In addition, the appropriate control procedures (e.g. yoked control experiments; see below) were also previously performed to show that our training schedules produce associative learning (Lukowiak et al., 2000, 2003b).

In experiments designed to test our 'residual memory trace' hypothesis (Figs 2–8), snails were given the ITM-training protocol on Day 1 (i.e. two 30 min training sessions with a 30 min interval between sessions). On the following day a second similar bout of ITM-training was given. The presence of LTM was tested 24 h later on Day 3. All experiments on memory retention were performed blind. The randomization of a cohort was performed by blindly separating the trained animals into two cohorts (i.e. testing one sub-cohort at 3 h and another at 24 h, etc.).

Yoked control procedure

These snails received a tactile stimulus to their pneumostome area that was not contingent upon opening their own pneumostome; rather they received the tactile stimulus when the snail to which they were 'yoked' to opened its pneumostome in the operant conditioning procedure (Lukowiak et al., 1996, 2003a). A similar intensity tactile stimulus was used as in the operant conditioning group. Since snails were most often underwater the 'yoked-poke' did not cause the pneumostome to close, as it was not open. We assayed the yoked control snails for memory 24 h after the last yoked control session. In the memory-test session these snails now received the tactile stimulus to the pneumostome when they attempted to open their pneumostome.

Cooling procedure

A 1 l beaker filled with 500 ml of eumoxic water was pre-chilled and maintained at 4°C and served as the cooling apparatus. We have previously shown that the cooling procedure does not adversely affect the snails (Sangha et al., 2003d). Cooling snails immediately (within 30 s) following operant conditioning training, reactivation of memory or extinction training, blocks the consolidation and re-consolidation processes (Sangha et al., 2003b,c,d). Therefore we test whether cooling snails immediately after the first bout of ITM-training can prevent the establishment of the residual molecular memory trace.

Cooling has also been used to extend the persistence of memory (Sangha et al., 2002d). Thus, if snails are permitted to undergo consolidation for ITM and then cooled, the duration

of the residual molecular memory trace should, if our hypothesis is correct, be enhanced. In these experiments snails were placed in the cooling apparatus 2 h after the last training session (i.e. after the consolidation process has been completed).

To control for any possible 'side-effects' of cooling a cohort of snails trained using the ITM-training protocol were kept at room temperature for 2 h. Following this 2 h period, snails were transferred into cold (4°C) eumoxic water for 46 h. Then snails were again trained using the ITM procedure at room temperature (20–23°C) and assayed for memory 24 h later (see Fig. 4).

ITM-training in a different context on day 2

In experiments designed to examine if the memory trace is context-specific, snails were given an ITM-training protocol in the standard context on Day 1. The following day, they were trained in a different context (i.e. the carrot context). One day later the snails were tested for memory (i.e. LTM) in the standard context. Our working hypothesis is that if the first bout of ITM-training is performed in one context, the residual memory trace will form for only that specific context. Thus, when the second bout of ITM-training is performed on the following day in a different context, there will be no LTM formed 24 h later when tested in the first context.

Extinction training protocol

Extinction training (for full details, see McComb et al., 2002; Sangha et al., 2003c) was performed by placing ITM-trained snails in a beaker of hypoxic water for two 30 min sessions separated by a 30 min rest interval in eumoxic water. During the 30 min extinction sessions the reinforcing stimulus (tactile stimulus to the pneumostome area) was not applied in response to a pneumostome opening. The two extinction sessions were given to the snails on Day 2. 3 h after the second extinction session, the second series of ITM-training was performed and memory was assayed 24 h later. In control experiments (Fig. 6B), different context (i.e. carrot) 30 min extinction sessions were given to snails. A bar labeled E1 or E2 denotes each extinction training session in the figures.

Somata ablation procedure

We have previously shown that the somata of RPeD1 is required for LTM formation, reconsolidation and extinction (Scheibenstock et al., 2002; Sangha et al., 2003b,c,e). The ablation procedure used here was performed as in our previous studies. Briefly, we first anesthetized the animals with 1–3 ml of 50 mmol l^{-1} $MgCl_2$ injected through the foot. This paralyzed the snail, allowing a dorsal midline incision to be made to expose the snail's brain. Using a fine glass hand-held microelectrode, the RPeD1 somata was ablated by gently 'poking' it. In control experiments, the somata of LPeD1, which is similar in size to RPeD1 but does not play a role in aerial respiratory behaviour, was ablated. The incision was small enough to allow the animal to heal without suturing. Animals began to wake from the effects of the anesthetic within several hours of the surgery. In the experiments reported here the experimenter performing the behavioural training was unaware of which neuron had been ablated. The code was only broken after the savings-test.

The ablation of RPeD1's somata, which leaves behind an intact functional primary neurite where the necessary synaptic interactions occur, does not adversely affect the snails' ability to perform aerial respiratory behaviour or to learn associatively (Scheibenstock et al., 2002; Sangha et al., 2003b,c). Total breathing time and the number of pneumostome openings were monitored before and after RPeD1 somata ablation. There were no significant differences between pre- and post-ablation in either measurement.

Criteria for learning and memory

We have operationally defined both associative learning and memory as previously (Lukowiak et al., 1996, 2003b; Sangha et al., 2003b,c). In the present study, learning on the particular day was considered present if the number of attempted pneumostome openings in the last training session (e.g. TS2) was significantly less than the number of attempted openings in the first training session (e.g. TS1). In order to be defined as memory, two criteria had to be met: (1) the number of pneumostome openings in MT was significantly lower than that of TS1, and (2) the number of pneumostome openings in the MT was not significantly higher than that of the last training session (e.g. TS2).

Statistical analysis

To determine whether the experimental manipulation had an effect when compared to a control group (see below) and whether the number of attempted pneumostome openings was significantly altered as a result of operant conditioning or other procedures (yoked control, cooling, extinction, etc.), we performed repeated-measures one-way ANOVAs, testing both a between-group factor (i.e. control *vs* experimental) and a within-group factor (i.e. training sessions *vs* savings-test; Zar, 1999). If the ANOVA was significant ($P<0.05$), a *post hoc* Fisher's LSD protected *t*-test was performed to show which groups (i.e. between group) and sessions (i.e. within group) were significantly different (Glass and Hopkins, 1996). Differences were considered to be significant if $P<0.05$.

Results

Demonstration of ITM and LTM with a different interval between training sessions

The experiments in Fig. 1A show the results of snails trained using the ITM-training procedure. Naïve snails ($N=89$) received two 0.5 h training sessions with a 0.5 h rest interval in between. As the number of attempted pneumostome openings in TS2 was significantly less than the number of attempted pneumostome openings in TS1 ($P<0.01$) we concluded that learning occurred ($ANOVA_{(88,3)}=40.1$, $P<0.0001$). We then tested a randomly picked cohort of these snails ($N=46$) for memory 3 h after TS2. Since there was

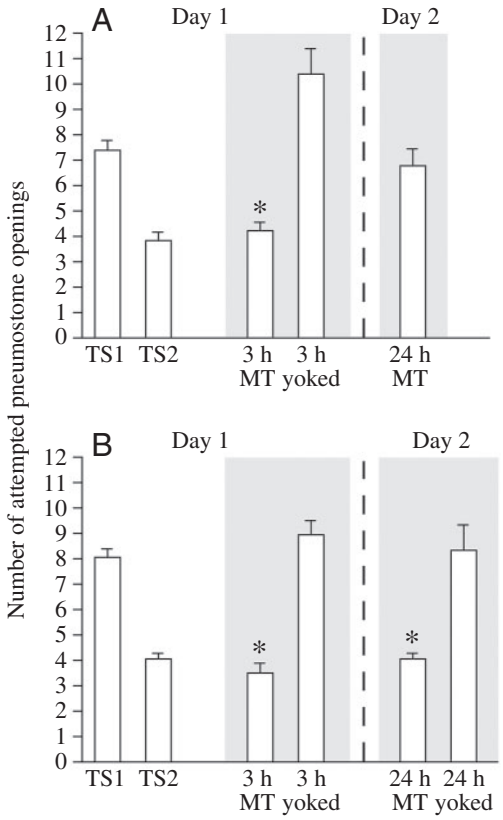

Fig. 1. Intermediate-term and long-term memory in *Lymnaea*. (A) An interval of 0.5 h between training sessions TS1 and TS2 results in an intermediate-term memory (ITM) that persists for 3 h but not 24 h as observed during memory tests (MT). (B) An interval of 1 h between the 30 min training sessions TS1 and TS2 results in a long-term memory (LTM) that persists for 3 h and at least 24 h after the last training session (TS2). Yoked controls (see text for details) in A and B did not show ITM or LTM. *Significant difference in number of openings from control TS1.

no significant difference in the number of attempted pneumostome openings between the 3 h MT session and TS2 (P>0.05) and since the number of attempted openings in 3 h MT was significantly less than the number in TS1 (P<0.01), we conclude that memory was present (i.e. the operational definition of memory was met). However, when we tested the remaining snails (24 h MT; N=43), 24 h after TS2 we found that memory was not present. That is, the number of attempted openings in the 24 h MT was a significantly greater than the number of attempted openings in TS2 (P<0.01). Furthermore, there was statistically no difference between TS1 and the 24 h MT session (P>0.05).

To conclude, however, that the change in behaviour was a true example of associative learning and memory formation, we performed yoked control experiments. In yoked control (N=28) snails (yoked to the ITM-training schedule) a memory test (Yoked MT) was performed 3 h after TS2. We found that the number of attempted openings in Yoked MT was not significantly less than the number of attempted openings in TS1 of the ITM-trained snails (P>0.05) and was statistically greater than the number of attempted openings in TS2 (P<0.01). We also made a between-group comparison of the response in MT in the yoked control and ITM operantly conditioned snails. We found that the number of attempted pneumostome openings in Yoked MT was significantly greater that the number of attempted openings in the 3 h MT (P<0.01) of operantly trained snails. We therefore concluded that two 30 min training sessions separated by an interval of 30 min results in ITM but does not in LTM.

By contrast, when a group of snails (N=155) was subjected to the LTM training procedure (two 30 min training sessions separated by a 1 h interval), ITM and LTM were observed (Fig. 1B), thus learning occurred ($ANOVA_{(154,3)}$=103.0, P<0.0001). The number of attempted openings in TS2 was significantly less than TS1 (P<0.01). When a randomly picked cohort of the snails was tested 3 h later (3 h MT; N=27) memory was exhibited. That is, there was no significant difference in the number of attempted openings between 3 h MT and TS2 (P>0.05) while the number of attempted openings in 3 h MT was significantly less than in TS1 (P<0.01). When the remaining snails (24 h MT; N=128), were tested for LTM 24 h after TS2, memory was also shown to be present. Thus there was no significant difference observed between 24 h MT and TS2 (P>0.05) and the number of attempted openings in 24 h MT was significantly less than the number in TS1 (P<0.01). Thus, the LTM-training procedure results in memory that persists for at least 24 h and can also be observed at 3 h (Fig. 1B).

Yoked control snails (to the LTM training procedure) received a memory test either 3 h or 24 h after TS2 (3 h Yoked MT; N=27; 24 h Yoked MT; N=28, respectively). Memory was not demonstrated in either session. We found that the number of attempted pneumostome openings in both MT sessions were not statistically different from TS1 (P>0.05), but were significantly greater than TS2 (P<0.01). Thus, two training sessions of non-contingent tactile stimuli to the pneumostome area with a 1 h interval between sessions did not result in LTM. We also made between-group comparisons of the response in MT in the yoked control and LTM operantly conditioned snails. We found that the number of attempted pneumostome openings of yoked control snails in Yoked 3 h MT and Yoked 24 h MT were significantly greater than (P<0.01) either the 3 h MT and 24 h MT sessions, respectively, of the LTM operantly trained snails.

We thus conclude that: (1) ITM and LTM can be differentially produced by altering the interval between training sessions; and (2) *Lymnaea* have the capacity of being operantly conditioned (i.e. associative learning) and forming LTM.

A second bout of ITM-training 24 h later causes LTM

We hypothesized that when we tested snails subjected to the ITM-training procedure for memory 24 h after TS2 (unsuccessfully, Fig. 1A) that we nonetheless caused the activation of a ‘molecular memory trace’ in neurons necessary for memory formation (e.g. RPeD1). We further hypothesized that this residual molecular memory trace could serve as a

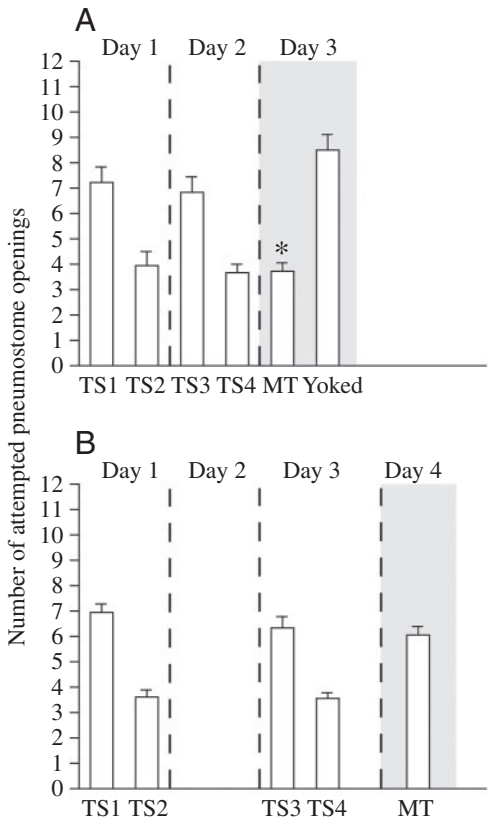

Fig. 2. 'Changing memory'. (A) A second bout of ITM-training (TS3 and TS4) 24 h after the first bout can cause LTM formation even if the behavioural phenotype of memory is absent. Yoked control snails (Yoked), given tactile stimuli at the same times as trained snails, did not exhibit LTM. (B) A 48 h interval between the two bouts of ITM-training does not result in LTM. *Significant difference in number of openings from control TS1 and TS3.

foundation upon which a second bout of ITM-training could produce LTM (i.e. there would be memory in a MT 24 h after the second bout of ITM-training).

We therefore subjected a naïve cohort of snails (N=37; Fig. 2A) to the ITM-training schedule on 2 consecutive days. Learning occurred on both days ($ANOVA_{(36,5)}$=20.0, P<0.0001); that is the number of attempted pneumostome openings in TS2 and TS4 were significantly less than the number in TS1 and TS3, respectively (P<0.01 in both cases). Two findings emerged from this experiment. The first finding, as expected, was that there was no memory on TS3. That is, the number of attempted pneumostome openings in TS3 was significantly greater than the number in TS2 (P<0.01), indicating that LTM was not formed. The second finding was that when we tested these snails for memory 24 h after a second bout of ITM-training (Day 3 MT), LTM was evident. That is, when memory was tested 24 h after TS4 (Day 3 MT), the number of attempted pneumostome openings was not statistically different from the number in TS4 (P>0.05), but was significantly different from the number in both TS1 and TS3, respectively (P<0.01 in both comparisons). Thus, the criteria for LTM were met.

Before we could conclude that there was a residual molecular memory trace present in neurons that could serve as a foundation on which to build a LTM memory with further ITM-training (i.e. TS3 and TS4), we had to perform a number of control experiments. The first was a yoked control experiment and a second was to increase the interval between the two ITM-training bouts from 24 h to 48 h.

To show that the LTM observed on Day 3 was not just the result of 2 days of receiving tactile stimuli, a yoked control procedure was used. When we subjected these yoked control snails (Day 3 Yoked; N=37) to a MT Session 24 h after TS4 we found that the number of attempted openings was not statistically different from either TS1 or TS3 of operantly conditioned snails (P>0.05), but was significantly different from TS4 (P<0.01). Most importantly, the number of attempted openings of yoked control snails in Yoked MT was significantly greater than the number of attempted openings in Day 3 MT (P<0.01) of operantly trained snails given the ITM-training procedure on two consecutive days. Thus, 2 consecutive days of non-contingent tactile stimuli (i.e. the yoked control procedure) to the pneumostome did not result in a change in aerial respiratory behaviour (i.e. memory was not observed).

We next imposed a 48 h interval between the two ITM-training bouts (Fig. 2B). Learning occurred on both days ($ANOVA_{(50,4)}$=27.2, P<0.0001). However, when we tested snails (N=77) for memory 24 h after TS4 we found that the criteria for memory were not met. That is, the number of attempted openings in MT was significantly greater than in TS4 (P<0.01) and was not significantly different from the number in either TS1 or TS3 (P>0.05 for both comparisons). We interpret these data in the following manner. The imposition of a 48 h interval between the two training bouts was sufficient to ensure that there was no vestige of a 'residual' memory trace on which to build an LTM memory. Thus we conclude that contingent presentation of a tactile stimulus to the pneumostome utilizing the ITM-training procedure is sufficient to result in a memory that lasts at least 24 h (i.e. LTM) if the second ITM-training sequence occurs within 24 h of the first ITM-training bout.

Cooling during the consolidation period blocks the residual memory trace

We have previously demonstrated that cooling snails immediately (i.e. within 30 s) after the last training session is sufficient to block the formation of either ITM or LTM (Sangha et al., 2003a). We therefore hypothesized that if we cooled snails immediately after TS2 on Day 1 there would be no vestige of the residual memory trace for the second bout of ITM-training given on Day 2 to build on, which would result in LTM. In Fig. 3A, a cohort of snails (N=24) received the ITM-training procedure on Day 1. Immediately after TS2 (i.e. within 30 s), snails were placed in water at 4°C for 2 h and then transferred to eumoxic room temperature water for 22 h. On Day 2, they again received the ITM-training procedure. Learning occurred on both days ($ANOVA_{(23,4)}$=7.76, P<0.0001); that is the number of attempted pneumostome

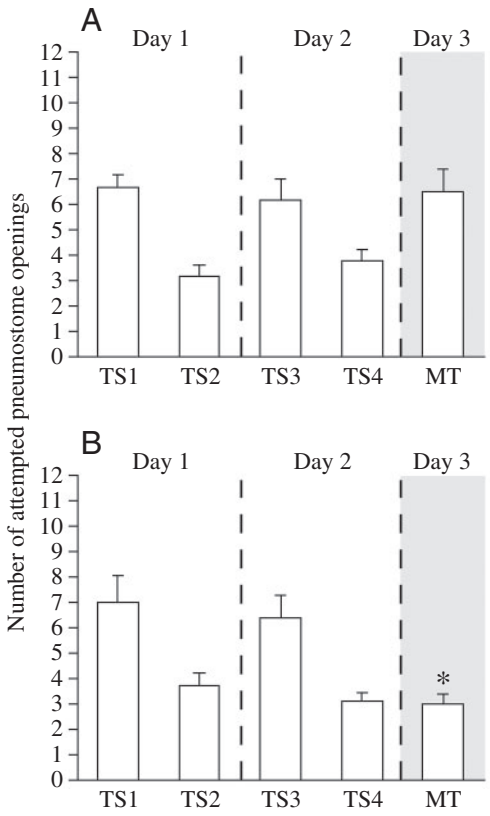

Fig. 3. Immediate cooling after training session 2 (TS2) blocks memory boosting. (A) Cooling snails immediately after ITM-training prevents the establishment of an ITM memory trace sufficient for a second bout of ITM-training to produce LTM. (B) When the cooling was administered after the consolidation process had occurred (i.e. 2 h after TS2) LTM was apparent 24 h later (MT). *Significant difference in number of openings from control TS1 and TS3.

openings in TS2 and TS4 were significantly less than the number in TS1 and TS3, respectively (P<0.01 in both cases). When memory (MT) was tested 24 h after TS4 on Day 3, it was not observed. That is, the number of attempted pneumostome openings in MT was significantly greater than the number in TS4 (P<0.01). Additionally, there was no significant difference between the number of attempted openings in MT and TS1 or TS3 (P>0.05). Thus LTM was not observed when snails were immediately cooled after TS2.

To control for the possible adverse effects of cooling on LTM memory formation, we performed the same experiment (Fig. 3B) as in Fig. 3A except the cooling was administered after the consolidation process had occurred (i.e. snails were cooled 2 h after TS2). The snails (N=22) were treated as in A, except that cooling was delayed for 2 h after TS2. Following their 2 h exposure to 4°C water they were returned to room temperature eumoxic pondwater for 20 h. Learning occurred on both days ($ANOVA_{(21,4)}$=10.9, P<0.0001). When we tested these snails for LTM 24 h after TS4 we found that LTM was present. That is, there was no significant difference between the number of attempted pneumostome openings in MT and TS4 (P>0.05), while there was a significant difference in the number between

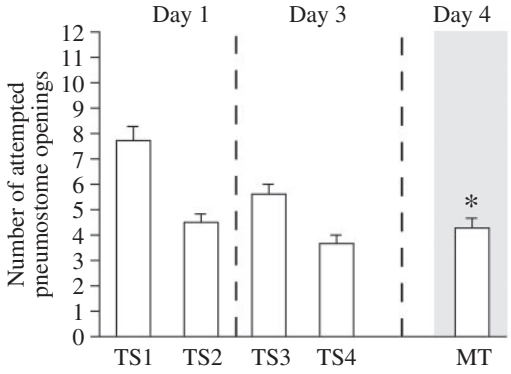

Fig. 4. Cooling applied after ITM consolidation has occurred extends the persistence of the residual memory trace for at least 48 h. Cooling snails to 4°C for 2 h after TS2 extends the period of time effective to produce LTM to 48 h between bouts of ITM-training. *Significant difference in number of openings from control TS1.

MT *vs* TS1 and TS3, respectively (P<0.01). We conclude that it is possible to interfere with the ITM consolidation process by cooling snails such that there is no memory trace to build an LTM memory on with subsequent ITM-training. Thus, LTM is not just the result of two consecutive days of ITM-training. Moreover the data are consistent with the hypothesis that a second bout of ITM-training is sufficient to produce LTM if there is a residual memory trace present in neurons that are necessary for LTM formation.

Cooling extends the memory trace and prolongs the interval between training

While immediate cooling following TS2 is able to block the consolidation process and thus prevent memory formation, cooling applied after consolidation paradoxically extends memory persistence (Sangha et al., 2003d). We therefore hypothesized that if we cooled snails for a period of 48 h after ITM-consolidation, a residual memory trace would still be present so that a second bout of ITM-training would result in LTM. Snails (Fig. 4) first received ITM-training on Day 1, and 2 h after TS2 they were placed in 4°C pondwater for 48 h. They then received a second bout of ITM-training (TS3, TS4). Learning occurred on both days ($ANOVA_{(37,4)}$=20.4; P<0.0001); that is, the number of attempted pneumostome openings in TS2 and TS4 were significantly less than the number in TS1 and TS3, respectively (P<0.01). When memory was tested 24 h after TS4 (MT), there was no significant difference between the number of attempted openings in MT and TS4 (P>0.05) while the number of attempted openings in MT was significantly less than both TS1 and TS3 (P<0.01). Thus memory was present. Thus cooling can preserve the residual memory trace so that a second bout of ITM-training 48 h after TS2 leads to LTM.

ITM-training followed by another ITM-training in a different context

Context-specific learning, memory formation and extinction

have all been demonstrated in *Lymnaea* (Haney and Lukowiak, 2001; McComb et al., 2002). We therefore hypothesized that a second bout of ITM-training would not result in the establishment of LTM, if the second bout of ITM-training was performed in a different context. Our reasoning was that the 'two different context ITM-training procedures' would result in two different associations (i.e. one ITM-memory for the standard context and another, different ITM-memory for the carrot context). Therefore, when we trained snails using the 'different context ITM-training' on Day 2 we would not be building on the residual memory trace that had been encoded in neurons for the standard context memory. Since no 'foundation' would be present, LTM for the standard context would not be observed 24 h later. As shown in Fig. 5A this is exactly what we found. A cohort of naïve snails (N=26) received ITM-training in the standard context. On Day 2, they again received ITM-training but this time in a different context (i.e. carrot; black shading indicates a carrot context was used and no shading indicates the standard context was used). Learning occurred on both days ($ANOVA_{(25,4)}$=22.0, P<0.0001). However, when we tested for LTM in the standard context 24 h after TS4 (Day 3 MT) LTM was not present. That is, the number of attempted openings in MT was significantly greater than TS2 (P<0.01) and the number of attempted openings in MT was not significantly different from TS1 (P>0.05). Although there was learning in both contexts, there was no LTM.

We also tested for the possibility that training in the standard context on Day 1 and then training in the carrot context on Day 2 produces a LTM on Day 3 for the carrot context. As shown in Fig. 5B, a cohort of naïve snails (N=27) received ITM-training in the standard context on Day 1. On Day 2, they received ITM-training in the carrot context. Learning occurred on both days ($ANOVA_{(26,4)}$=14.5, P<0.0001). When we tested for memory (MT) in the carrot context 24 h after TS4, LTM was not present. That is, the number of attempted openings in MT was significantly greater than in TS4 (P<0.01) while there was no significant difference between MT and TS3 (P>0.05).

Similar results were found if we reversed the presentation of the contexts used for ITM-training (Fig. 5C; i.e. carrot first, standard second). While learning occurred on both days ($ANOVA_{(27,4)}$=16.9, P<0.0001) LTM was not shown. That is the number of attempted openings in MT was significantly greater than in TS2 (P<0.01) and there was no significant difference between MT and TS1 (P>0.05). All these data demonstrate that it was not simply the 'extra' application of tactile stimuli, even contingent stimuli that lead to LTM formation. Rather, the 'extra' training must be within the same context in order to build upon a residual memory trace.

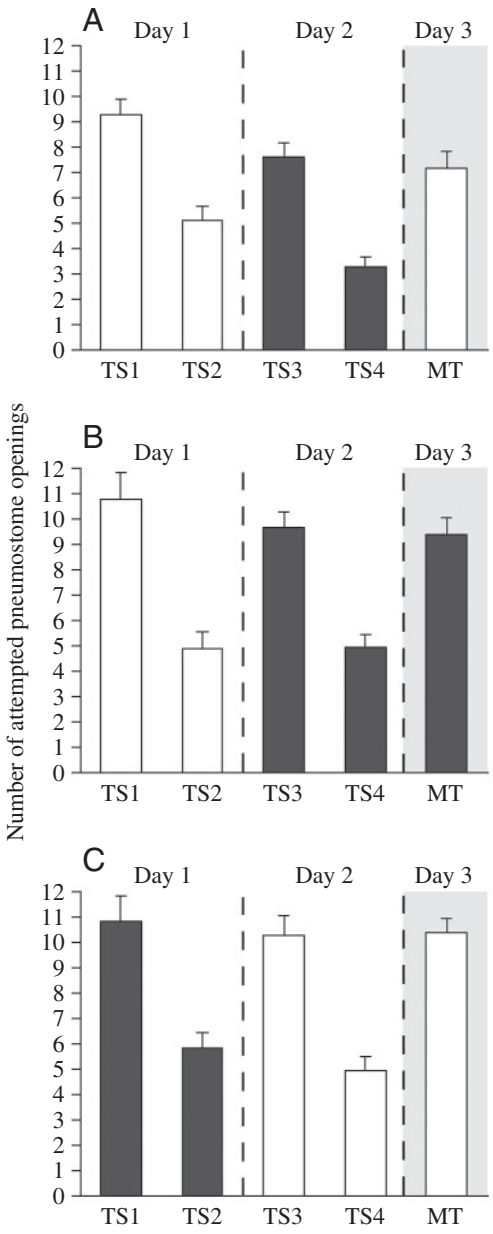

Fig. 5. Change of context and memory boosting. (A) A change in context on Day 2 ITM-training prevents the establishment of LTM. White bars, standard training context; black bars, carrot training context. See text for details. (B) Training in the standard context on Day 1 and then training in the carrot context on Day 2 does not produce a LTM on Day 3 in the carrot context. (C) Similar results were found as in B if we reversed the presentation of the contexts used for ITM-training.

ITM-training followed by extinction training and further ITM-training

We have demonstrated in *Lymnaea* that extinction training results in a new memory that co-exists with but occludes the old memory (Sangha et al., 2003c). We therefore hypothesized that the imposition of extinction training between the two ITM-training bouts would prevent the subsequent formation of LTM. We reasoned that extinction training would occlude or make inaccessible the memory trace produced by the first bout of ITM-training and thus the second bout of ITM-training (following the extinction training) would not have a foundation (i.e. residual memory trace) on which to build to produce LTM. That is, LTM would not be observable in MT 24 h after Session 4.

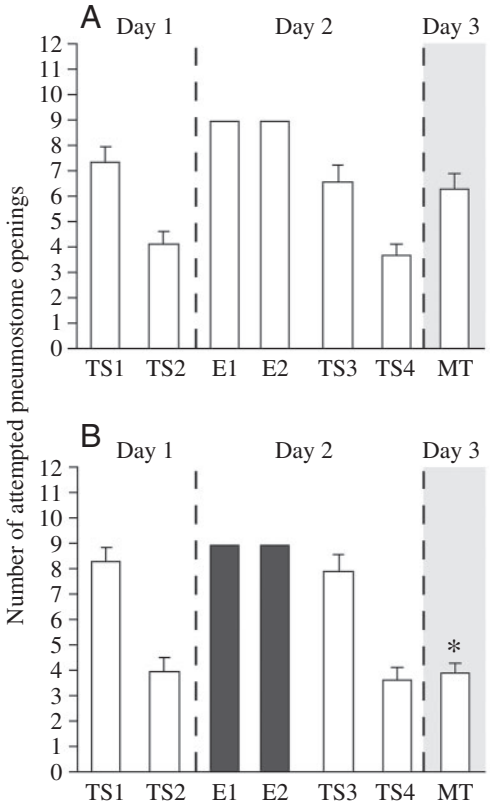

Fig. 6. Extinction training and the ability to 'boost' memory. (A) Extinction training (E1 and E2) in the same context (hypoxic water; white bars) prevents LTM formation. (B) Extinction training in a different context (carrot-odorant containing hypoxic water; black bars) allows LTM formation. See text for details. *Significant difference in number of openings from control TS1 and TS3.

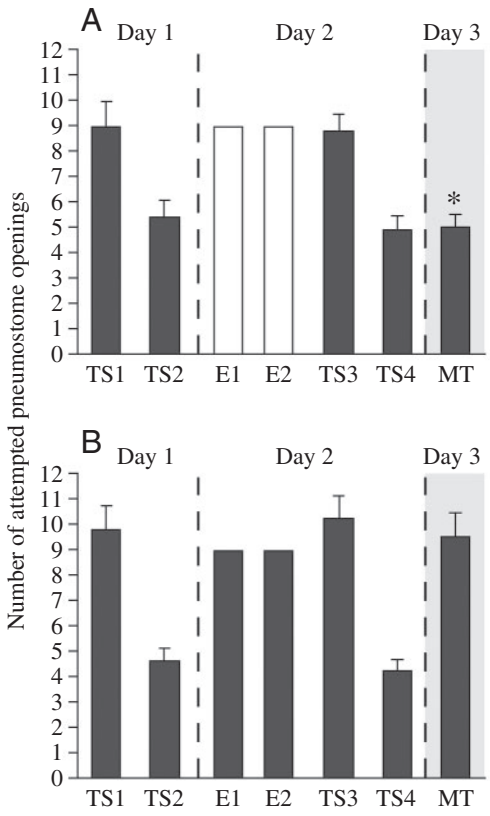

Fig. 7. Altering the sequence of operant conditioning and extinction training in different contexts did not alter the observed results. (A) The interposition of extinction training (E1 and E2) in the standard context (white bars) on Day 2 did not prevent LTM formation as a result of a second bout of ITM-training in a carrot context (black bars). (B) The interposition of extinction training in the carrot context on Day 2 prevents LTM formation as a result of a second bout of ITM-training in a carrot context. *Significant difference in number of openings from control TS1 and TS3.

A cohort of naïve snails (Fig. 6; N=32) on Day 1 received ITM-training. On Day 2, these snails first received a bout of extinction training (i.e. two 0.5 h sessions separated by a 0.5 h interval, in which they were placed in the hypoxic environment but did not receive the reinforcing stimulus when they opened their pneumostome; E1 and E2). 3 h later they received a second bout of ITM-training. Learning occurred in both ITM-training sessions ($ANOVA_{(31,4)}$=10.6, P<0.0001). However, when we tested for memory 24 h after TS4, LTM was not present. That is, the number of attempted openings in MT was significantly greater than in TS4 (P<0.01) and there was no significant difference between MT and TS1 or TS3 (P>0.05). Thus, the interposition of extinction training prevented the second bout of ITM-training to produce LTM.

We have previously shown that extinction is also context-dependent (McComb et al., 2002). We reasoned that subjecting snails to a 'different context extinction training procedure' (i.e. 'carrot-extinction') would not occlude the memory trace for the standard context. This experiment (Fig. 6B) would also serve to control for the possible time effects of the interposed extinction sessions on the formation of LTM. The experiment (N=26) followed the same protocol as in A except that now the extinction training was performed in the 'carrot-context' (black shaded E1 and E2 bars). Learning occurred on both days ($ANOVA_{(25,4)}$=23.3, P<0.0001). However, when we tested for memory 24 h after TS4, LTM was present. That is, the number of attempted openings in MT was not significantly greater than in TS4 (P>0.05) but was significantly less than either TS1 or TS3 (P<0.01). Thus, the interposition of extinction training in a different context did not prevent the formation of LTM.

We repeated the experiments shown in Fig. 6 this time using the carrot context for operant conditioning training and either the 'carrot' or 'standard context' for the extinction training (Fig. 7), and obtained similar results. If a different context extinction training procedure was used (i.e. the standard context) LTM was formed (Fig. 7A), while the interposition of extinction training in the same context (i.e. carrot) prevented LTM formation (Fig. 7B).

A cohort of naïve snails (N=26) received the ITM-training procedure in the carrot-context on Day 1. On Day 2, they received extinction training (E1 and E2, white bars) in the standard context, followed 3 h later by the ITM-training procedure in the carrot context (black bars). Learning occurred

on both days ($ANOVA_{(25,4)}$=17.2, P<0.0001). LTM was present when the interposed extinction training was performed in the standard context [Fig. 7A; i.e. there was no significant difference between the number of attempted openings in MT and TS4 (P>0.05) while MT was significantly less than both TS1 and TS3, respectively (P<0.01)]. By contrast, LTM was not present if the interposed extinction was performed in the carrot context [Fig. 7B; i.e. the number of attempted openings in MT was significantly different from TS4 (P<0.01) and was not significantly different from either TS1 or TS3 (P>0.05)].

Together the results in Figs 6 and 7 show that it is not simply a second bout of ITM-training 24 h after the first ITM-training sessions that results in LTM formation. The residual memory trace, which can be occluded by extinction training in the same but not a different context, has to be present in order for LTM to be produced.

The residual memory trace can lead to LTM formation only if somata of RPeD1 are present

Previously we have shown that the somatata of RPeD1 must be present for LTM formation, extinction and memory reconsolidation (Scheibenstock et al., 2002; Sangha et al., 2003b,c). Thus, we hypothesized that ablation of RPeD1's somata before ITM operant conditioning training would prevent a residual memory trace from forming a foundation for LTM with subsequent ITM-training. That is, because LTM requires gene transcription and since somata ablation removes the nucleus and thus the genes, LTM formation should not occur. These data are shown in Fig. 8. A cohort of naïve snails had the somata of either RPeD1 (N=19) or LPeD1 (N=9) ablated 2 days before ITM-training. All snails received two bouts of ITM-training 24 h apart. Learning occurred on both days ($ANOVA_{(36,5)}$=17.7, P<0.0001). When memory was tested (MT) 24 h after TS4, we found that snails with RPeD1 somata ablated, behaved differently than those with LPeD1 somata ablated. In the LPeD1 cohort (L-MT) there was LTM. That is, the number of attempted pneumostome openings in L-MT was not significantly different than the number in TS4 (P>0.05) but was significantly less than in TS1 and TS3 (P<0.01). On the other hand, LTM was not present in the RPeD1 somata-ablated cohort (R-MT). That is, the number of attempted pneumostome openings in R-MT was significantly greater than the number in TS4 (P<0.01) and was not significantly different than the number in either TS1 or TS3 (P>0.05). Thus, we conclude that the somata of RPeD1 must be present in order for the second series of ITM-training to produce LTM.

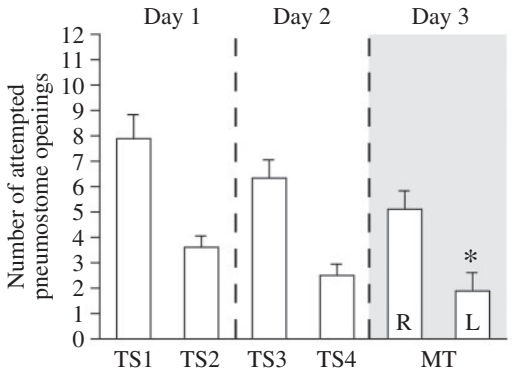

Fig. 8. The somata of RPeD1 is necessary for memory boosting. Snails had either the somata of RPeD1 (R) or LPeD1 (L) ablated and were given 2 days to recover from the procedure. Both cohorts were given 2 days of ITM-training (TS1, TS2, TS3 and TS4) but only the snails that had the somata of LPeD1 ablated were able to form LTM. RPeD1 somata ablation but not LPeD1 somata ablation prevented memory 'boosting'. *Significant difference in number of openings from control TS1 and TS3.

Discussion

Previously it was shown (Lukowiak et al., 2000) that in *Lymnaea* with the conditioning of aerial respiration there are 2 forms of memory lasting more than a few minutes, ITM (2–3 h persistence) and LTM (>6 h persistence). LTM is dependent on both transcriptional and translational processes, while ITM is only dependent on the translational process (Sangha et al., 2003d). In addition, somata of RPeD1 must be present for learning to be consolidated into LTM, but these are not required for the formation of ITM (Scheibenstock et al., 2002). RPeD1's somata must also be present for the process of reconsolidation following activation of the memory (Sangha et al., 2003b). This reconsolidation process requires both altered gene activity and new protein synthesis. Thus, when an attempt is made to retrieve a memory, the underlying causal molecular processes shift from a stable state to an active, labile state. In order for the memory to be stabilized again it goes through the reconsolidation process (Nader, 2003).

We show here that following an ITM-training procedure the attempt to retrieve the memory 24 h later (futile at the behavioural level) causes a 'residual molecular memory trace' in a neuron, RPeD1, necessary for LTM formation. Activation of this trace is sufficient to enable a second bout of ITM-training to produce LTM. We call this phenomenon 'memory boosting'. However, memory boosting only occurs if: (1) RPeD1's somata are present; (2) the second bout of ITM-training occurs within 24 h of the first series; (3) the initial learning undergoes consolidation into ITM; and (4) the second bout of ITM-training occurs in the same context as the first. Finally, extinction training in the same context interposed between the two bouts of ITM-training is capable of occluding or preventing the residual memory trace from being activated and thus prevents LTM formation. Alternatively, perhaps during extinction the residual memory trace is being activated and altered to favor a CS-no US association (non-reinforced association).

These data are all consistent with the hypotheses that: (1) memory exists in either an active (labile) or an inactive (stabile) state and that following its activation it returns to the stabile state in a transcription- and translation-dependent manner; (2) retrieval of ITM causes activation of a molecular memory trace which can in some circumstances form a

foundation in which a second ITM-training bout causes LTM to be formed; and (3) the molecular processes underlying LTM build on the molecular processes that cause ITM.

The primary question asked here was 'Does the attempted retrieval of an ITM memory, even if behaviourally memory is not apparent, result in the formation of a molecular memory trace?' We earlier showed that previous ITM-training potentiates the duration of LTM produced by subsequent LTM-training, hypothesizing that potentiation was due to a facilitating priming effect of the residual ITM trace (Smyth et al., 2002). We found in the present work that activating the ITM 24 h, but not 48 h, after the last ITM-training session was sufficient to allow a second bout of ITM-training to produce LTM (Fig. 2). Neither yoked controls nor snails that received ITM-training in two different contexts 24 h apart subsequently exhibited LTM. Moreover, if the ITM consolidation process (i.e. the molecular memory trace) was blocked by immediate cooling, a second bout of ITM-training failed to produce LTM. Thus, LTM formation following a second bout of ITM-training was not just the result of another session of ITM-training. To be successful in producing LTM, the second bout of ITM-training had to build on the presence of a residual memory trace created by the initial ITM-training bout. The absence of LTM found in the yoked control snails further shows that it is only contingent reinforcement using an ITM-specific training procedure that leads to LTM formation.

However, a second bout of contingent ITM-training was not by itself sufficient to cause LTM formation; the second bout had to be in the same context as the first ITM-training. Snails show context-dependent memory (Haney and Lukowiak, 2001), thus when a different context training regimen is used in the second bout of ITM-training, LTM is not produced because there is no residual memory trace for the new context to build upon. The hypothesized residual memory trace could also be 'interfered with' in a number of ways. (1) Increasing the time interval to 48 h between ITM-training bouts, (2) extinction training in the same context between training bouts, and (3) cooling to 4°C immediately after Session 2 to prevent ITM formation and thus a molecular memory trace.

We have previously shown that the immediate cooling of snails to 4°C following training blocks the ITM consolidation process (Sangha et al., 2003d). Thus we reasoned that the immediate cooling of the snails would block the formation of the 'ITM molecular memory trace' and thus prevent the second bout of ITM-training from producing LTM. Cooling, however, was only effective in blocking the production of LTM by the second bout of ITM-training if given immediately after TS2. If applied 2 h after TS2, cooling had no interfering effect. In fact, if cooling is applied after consolidation it prolongs the persistence of the ITM molecular memory trace (Sangha et al., 2003d) and thus extends the effective time interval between the two ITM-training bouts that lead to the formation of LTM. We have not yet determined how long we can extend the persistence of the residual memory trace by cooling. We are also uncertain what cellular processes cause the ITM molecular memory trace to become ineffective as a foundation for a second bout of ITM-training to build upon to produce LTM, if the interval between training bouts is increased from 24 h to 48 h. One hypothesis is that interfering behavioural events (e.g. spontaneous aerial respiration) that occur without reinforcement result in a 'spontaneous extinction' memory that occludes the memory trace. We are currently attempting to test this hypothesis directly.

We also asked whether the LTM generated by the second series of ITM-training is dependent on altered gene activity (i.e. transcription). In *Lymnaea,* as in other organisms, LTM is dependent on transcription and translation (Dudai, 2002b; Scheibenstock et al., 2002; Sangha et al., 2003a). A major advantage of the *Lymnaea* model system is that it is possible to surgically remove the somata of RPeD1 in an otherwise intact naïve snail, and show that while learning and ITM occur, LTM formation does not because there is no nucleus (i.e. no genes; Scheibenstock et al., 2002). We found in RPeD1 somata-less snails that a second series of ITM-training did not result in LTM. The translation of proteins necessary for ITM in these preparations occurs extra-somally, but the second bout of ITM-training cannot result in LTM because the nucleus is absent and the transcription of mRNAs necessary for LTM cannot transpire. Thus, we conclude that the LTM we observe following the second bout of ITM-training is dependent on transcription in RPeD1 in addition to translation of new proteins.

Our working hypothesis is that LTM formation is at least a two-step serial process. The first step parallels ITM formation and only requires new protein synthesis, which may occur extra-somally (Scheibenstock et al., 2002; Spencer et al., 2002; van Minnen et al., 1997). These new proteins may serve to mark the site for subsequent events necessary for LTM formation, as suggested in *Aplysia* (Martin et al., 1997). The second phase of LTM formation requires the transcription of genes, but may not involve translation of those mRNA transcripts within the somata. Ultimately these new proteins arrive at the ITM site of encodement (e.g. a presynaptic terminal) to create LTM. Previously we found that LTM was only observed if we employed a training regimen that consisted of a single 1 h training session or had a 1 h interval between training sessions (Lukowiak et al., 2000; Sangha et al., 2003a,d). We hypothesize that the shorter interval (0.5 h *vs* 1.0 h) between training sessions does not promote the downregulation of the suppressive cAMP response element binding protein (CREB) isoform, and therefore the altered gene activity (i.e. transcription) necessary for LTM is not initiated. The ratio of CREB activator to repressor isoform has been considered as a 'molecular switch' to initiate the processes that cause LTM formation (Sutton et al., 2002). Thus, intervals or events that alter the ratio in favor of the activator isoform would lead to LTM formation. However, a recent report suggests that it is the downregulation of the suppressor isoform of CREB that is the most important factor initiating the molecular cascade leading to LTM formation (Perazzona et al., 2004). Here we suggest that the first bout of ITM-training results in the establishment of a memory trace in RPeD1 (and in other neurons necessary for LTM formation), permitting a

subsequent ITM-training bout to induce the necessary transcription factors for LTM even if the behavioural manifestation of memory was not apparent. Hence, our use of the terms 'residual memory-trace' and 'memory boost' for the phenomena described here. Our findings are similar in this respect to the reports that the prior induction of molecular factors necessary for LTM allowed training procedures, which characteristically do not produce LTM, to cause LTM (e.g. Müller, 2000; Yin et al., 1995; but see Perazzona et al., 2004).

A two-step LTM formation process, with the first step matching the processes that underlie ITM formation, is consistent with other reports. For example, in the crab *Chasmagnathus*, two phases of cAMP-dependent protein kinase (PKA) activation are needed for learning to be consolidated into LTM. The first phase occurs during training and another phase occurs 48 h after training. This suggests that PKA may play a role in establishing a memory trace and its activation leads to LTM (Locatelli et al., 2002). Using contextual fear conditioning with weak training in mice, researchers have been able to show that LTM consolidation also has two phases that are sensitive to PKA and protein synthesis inhibitors (Bourtchouladze et al., 1998). There has been debate about the interrelated nature of the different forms of memory, as to whether they occur in parallel (Crow et al., 2003; DeZazzo and Tully, 1995; Emptage and Carew, 1993; Hegde et al., 1997; Izquierdo et al., 2002; Mauelshagen et al., 1996; Tully et al., 1994) or in series (Ghirardi et al., 1995; Riedel, 1999; Sutton et al., 2001; Zhao et al., 1995). Conjoint serial and parallel processing of memories are also possible (Sutton et al., 2002). Our data clearly show that the processes that cause ITM formation have the capacity to permit LTM formation following a second series of ITM-training even if the behavioural phenotype of memory is absent. Nonetheless, the processes underlying LTM formation are clearly different from those underlying ITM, as the somata of RPeD1 are necessary for our training regimen to produce LTM but not ITM. However, the establishment of a memory trace by an ITM-training protocol can lay a foundation for subsequent training to produce LTM. This suggests to us that a molecular memory trace is laid down as a consequence of ITM activation, which serves as a permissive substrate, sufficient to allow the necessary transcription and translation that is causal for LTM formation.

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