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Intermediate and long-term memories of associative learning are differentially affected by transcription *versus* translation blockers in *Lymnaea*

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Summary

Aerial respiratory behaviour in the pond snail, *Lymnaea stagnalis*, can be operantly conditioned. This associative learning then undergoes consolidation into a long-lasting memory which, depending on the training procedure used, causes intermediate-term memory (ITM; lasting 3 h) or long-term memory (LTM; lasting >6 h) to be formed. We determined the differential susceptibility of these two forms of memory to translation and transcription blockers. The injection of a translation blocker, Anisomycin, 2.5 h before training prevents the establishment of both ITM and LTM. On the other hand, injection of the transcription blocker Actinomycin D, 2.5 h before training, did not prevent the establishment of ITM, but did, however, prevent LTM formation. Thus in *Lymnaea*, following associative learning, both ITM and LTM are dependent on new protein synthesis. ITM appears to be dependent on protein synthesis from pre-existing transcription factors, whilst LTM is dependent on protein synthesis from new transcription messages.

Key words: *Lymnaea stagnalis*, intermediate memory, long-term memory, protein synthesis, associative learning.

Introduction

Memories are vulnerable to disruption following a learning experience until they become stabilized through processes beginning during the learning phase and extending for a few hours afterward. That is, memory is labile until it undergoes the consolidation process (Lechner et al., 1999; McGaugh, 2000). Memory can be characterized in a number of different ways, one being for how long the memory persists. Thus memory can be divided into short (STM), intermediate (ITM) and long-term (LTM) forms. STM persists for only a few minutes while the longer lasting ITM and LTM persist for hours, days, weeks and years. ITM endures for only a few hours, while LTM survives for at least 1 day. The cellular, biochemical and molecular differences underlying these two forms of long-lasting memory are not completely understood (Squire and Kandel, 1999; Kandel and Pittenger, 1999). Most of the recent studies on formation and persistence of memory have focused on neural analogues of STM and LTM (Lechner et al., 1999; Martin et al., 2000). At both the behavioural and neuronal levels, however, far less attention has been paid to the shorter-lasting form of LTM, which was termed ITM in 1993 by Rosenzweig et al. (1993) (Lukowiak et al., 2000; Sutton et al., 2001).

The variation in the length of perseverance between ITM and LTM is most likely due to important molecular dissimilarities that underlie their encodement. Chief among these differences is the necessity for the transcription process. While both ITM and LTM require new protein synthesis, only LTM requires the transcription process. That is, LTM requires both altered gene activity and protein synthesis, while ITM requires only the translation process (Davis and Squire, 1984; Rosenzweig et al., 1993; McGaugh, 2000). Data obtained so far strongly support the idea that there are evolutionarily conserved mechanisms underlying LTM formation. They appear to involve both a cAMP-dependent MAP kinase signal transduction cascade culminating in the activation of CREB transcription factors (Tully, 1998; Mayford and Kandel, 1999; Silva et al., 1998) and formation of the CCAAT enhancer binding protein (C/EBP; Alberini et al., 1994; Taubenfeld et al., 2001).

Far less is known about the molecular basis underlying ITM. Prior to the discovery of a memory component of intermediate duration dependent upon different classes of protein kinase activities than those required for LTM (Rosenzweig, 1993), it was widely believed that ITM was indistinguishable from LTM. These shorter-lasting forms of memory have since been distinguished at a behavioural level through classical conditioning of *Aplysia* feeding behaviour (Botzer et al., 1998) and sensitization of the siphon withdrawal response (a form of non-associative learning; Sutton et al., 2001), as well as through operant conditioning of aerial respiration in *Lymnaea* (Lukowiak et al., 2000). At the neuronal level, analogues of ITM have been demonstrated at both *Aplysia* and *Hermissenda* CNS synapses where synaptic transmission is facilitated (Ghirardi et al., 1995; Crow et al., 1999; Sutton et al., 2001).

This form of synaptic facilitation requires protein synthesis but, unlike neuronal analogues of LTM, does not require transcription, suggesting that the proteins necessary for ITM formation are translated from pre-existing mRNAs.

A major advantage of conditioning aerial respiratory behaviour in *Lymnaea* is that the neural circuitry controlling this behaviour is well established. A three-neuron central pattern generator (CPG), which is both necessary and sufficient, mediates aerial respiration (Syed et al., 1990, 1992). In the operant conditioning procedure, snails associatively learn not to perform aerial respiration as a result of the contingent presentation of a tactile stimulus to their respiratory orifice, the pneumostome, each time they attempt to open it. Since *Lymnaea* are bimodal breathers, satisfying their respiratory needs *via* cutaneous and/or aerial respiration, we are able to perform experiments in which aerial respiratory behaviour is prevented or compromised without harming them (Lukowiak et al., 1996; Taylor and Lukowiak, 2000). Neural correlates of this operant conditioning have been demonstrated in the CPG neurons in both isolated ganglia and semi-intact preparations (Spencer et al., 1999, 2002). A second advantage is that by modifying the interval between training sessions, the training session duration, or the number of training sessions per day, ITM *versus* LTM can be differentially produced (Lukowiak et al., 2000). Given these advantages we have begun to ask how memory of the associative learning is encoded within this prescribed neuronal network. One necessary step on this path is to determine whether the different forms of memory (ITM and LTM) in *Lymnaea* are differentially affected by translation *versus* transcription protein synthesis blockers, as in other systems studied to date.

To accomplish this task we examined whether a translation blocker, Anisomycin, and a transcription blocker, Actinomycin D, differentially affect ITM and LTM. Anisomycin and Actinomycin D have previously been used in studies to examine their effects on memory consolidation in molluscs as well as other animals, including mammals (Castellucci et al., 1988; Nguyen et al., 1993; Milner et al., 1998; Ramirez et al., 1998; Crow et al., 1999). We have also used both of these blockers previously in *Lymnaea* to demonstrate the necessity for altered gene activity and new protein synthesis in synaptogenesis as well as to show that isolated primary neurites (i.e. without the soma) have the capacity to translate mRNA into functional membrane proteins (Feng et al., 1997; Woodin et al., 1999; van Minnen et al., 1997; Spencer et al., 2000).

Materials and methods

Animals

Laboratory raised *Lymnaea stagnalis* L., a freshwater pond snail, were maintained in aerated aquaria at room temperature (23°C) in the snail facility at the University of Calgary. Snails with volumes of 3 ml were used, which corresponds to a shell length of approximately 20 mm. Snails were fed *ad libitum* on lettuce.

Training procedures

Individually labeled snails were placed in a 1 l beaker containing 500 ml of hypoxic water. The water was made hypoxic by bubbling N_2 through it for 20 min prior to and during training. We refer to this as the ‘standard’ hypoxic training procedure. We also utilized a ‘different context’ training procedure, which we will refer to as the ‘carrot context’. To create the carrot context, N_2 was first bubbled through a 750 ml Erlenmeyer flask containing chopped carrots and water before being bubbled into the training beaker (for complete details, see Haney and Lukowiak, 2001). ‘Change of context test’ refers to the context in which the snails were **not** trained. This test was used as a control to show that, following a given procedure, e.g. training in the presence of a protein synthesis blocker, snails were still as responsive as they had been in the initial training session.

In all experiments, the snails were first given a 10 min acclimatization period, where they could perform aerial respiration freely. The onset of operant conditioning training was initiated by gently pushing the snails beneath the water surface. In between the training and memory test sessions, snails were placed in eumoxic aquaria where they were also allowed to perform aerial respiration freely.

In all of the training, memory test, and change of context test sessions, a gentle tactile stimulus (a sharpened wooden applicator) was applied to the pneumostome area (the respiratory orifice) every time the snail began to open its pneumostome to perform aerial respiration. This tactile stimulus only evoked pneumostome closure; it did not cause the animal to withdraw its foot and mantle area (i.e. the whole-animal withdrawal response), nor did pneumostome stimulation cause the snails to sink to the bottom of the beaker. The time of each attempted opening was recorded and tabulated.

Intermediate and long term memory training procedures

Snails can be differentially trained to produce ITM or LTM (Lukowiak et al., 2000). We used similar training procedures here. For ITM, snails were subjected a single 30 min training session, and memory was tested 3 or 4 h later in a 30 min test session.

For LTM, snails received a single 1 h training session, and memory was tested 6 or 24 h later in a 1 h session.

Following each respective training session we summed the number of attempted pneumostome openings for each animal and calculated the mean and the standard error of the mean (S.E.M.) of the total number of attempted pneumostome openings for each cohort.

A memory or ‘savings’ test session was presented to the snails at the indicated times. This test session was the same as a training session and was performed at varying times after the training session in order to determine how long the memory persists.

The ‘carrot-context’ procedure was used in some experiments. Snails demonstrate context-specific learning and memory (Haney and Lukowiak, 2001; McComb et al., 2002),

so it was possible to determine if memory was present, or if the observed behavioural phenotype was the result of an unresponsive animal due to drug-induced side effects, by altering the context in which we tested the snails. If the animal was unresponsive due to sickness, altering the context of the memory test session would not result in an increase in the number of attempted pneumostome openings. If the observed behaviour was the result of memory, however, altering the context would result in an increase in responsiveness.

Injections

The protein synthesis blocker (dissolved in saline) or saline control was injected into the hemocoel through the foot of the animal. The person injecting the drug or saline did not perform the training or the memory tests and the experimenter performing the training/memory test did not know what each snail had been injected with. Thus all experiments are performed 'blind'. The concentrations used were 12.5 μg Anisomycin ml^{-1} snail volume and 1 μg Actinomycin D ml^{-1} snail volume, which were the same as those used effectively in our laboratory to block both the transcription and translation processes, respectively (Feng et al., 1997; van Minnen et al., 1997; Hamakawa et al., 1999). We also directly demonstrated elsewhere that each of these blockers inhibits protein synthesis (van Minnen et al., 1997; Feng et al., 1997).

Results from pilot studies showed that these concentrations of protein synthesis blockers were effective when injected into the whole animal. We recalculated the concentrations so that an amount of 0.1 ml could be injected into snails of 3 ml total volume.

Operational definitions of learning and memory

We have operationally defined memory in previous publications (e.g. Lukowiak et al., 1996, 1998, 2000; Spencer et al., 1999, 2002). Memory was present if the number of attempted pneumostome openings in the memory test session was significantly less than the number of attempted openings in the training session. ITM was tested 3 and 4 h after the single 30 min training session, whilst LTM was tested 6 h after the last training session. Previously it was shown that ITM did not persist for longer than 3 h (Lukowiak et al., 2000). We had to test for LTM at 6 h post-training because control snails injected with Actinomycin D showed drug-induced side effects after this time (see below).

Statistical analysis

To determine whether there were any detrimental side-effects on aerial respiratory behaviour, data from snails injected with the protein synthesis blockers were subjected to a one-way analysis of variance (ANOVA) followed by a *post hoc* Fisher's LSD protected *t*-test to compare each session. A paired *t*-test (between groups) was used to determine whether memory was present (see above). A paired two-sample *t*-test for independent groups was used to compare the number of pneumostome openings in the first session of the different ITM and LTM training cohorts (i.e. control *versus* treatment with either of the two blockers). Significance was at the $P<0.05$ level.

Results

Effect of protein synthesis blockers on breathing behaviour

Before we could confidently use either of the two blockers to examine their respective effects on memory consolidation we had to determine whether they caused any potential deleterious side effects on aerial respiratory behaviour in a hypoxic environment. Anisomycin (12.5 μg ml^{-1} snail volume) or Actinomycin D (1 μg ml^{-1} snail volume) were injected into the hemocoel of snails through the foot. Pilot experiments demonstrated that higher concentrations of the blockers resulted in sickness of the snails and alterations in aerial respiratory behaviour, and we determined the time window in which we could safely study the specific effect of the blockers on the memory consolidation process (Fig. 1).

Snails were observed at 2.5, 4.5, 8 and 24 h following injection. Snails were divided into four groups. One (N=20) served as non-injected controls, while the other groups were injected with saline (N=20), Anisomycin (N=25) or Actinomycin D (N=22), respectively (Fig. 1). The injections and observations were performed by different people, therefore assessment of the snails' breathing behaviour in this and all other experiments reported here was performed 'blind'.

We examined whether the various injections significantly affected either the number of pneumostome openings or the duration of each opening by comparison with those of the non-injected snails. We thus calculated and tabulated the mean of the total breathing time (Fig. 1A) and the mean number of openings (Fig. 1B) per 30 min observation session per group.

In the non-injected controls, the total breathing time (ANOVA, $F_{3,19}$=0.5407, P=0.6564) and the number of pneumostome openings (ANOVA, $F_{3,19}$=0.5816, P=0.6295) did not change significantly over the four sessions. Therefore, repeated sequential exposure to hypoxic conditions did not alter the breathing behaviour of *Lymnaea*. Similarly, no significant differences in total breathing time (ANOVA, $F_{3,19}$=0.1093, P=0.9539) or the number of pneumostome openings (ANOVA, $F_{3,19}$=0.5247, P=0.6671) were detected in saline-injected snails over the four hypoxic water test sessions. Vehicle injection did not significantly change either the total breathing time or the number of breaths when we compared the saline and non-injection groups (paired *t*-test: $P>0.05$).

Injection of the translation blocker Anisomycin did not significantly affect either the total breathing time or the number of pneumostome openings (ANOVA, $F_{3,24}$=0.6114, P=0.6112 and $F_{3,24}$=0.259, P=0.847, respectively) at any of the time points. Comparison of the Anisomycin-injected group with the non-injected group showed that there was not a significant difference in total breathing time or in the number of pneumostome openings (paired *t*-test, $P>0.05$). We concluded therefore that when studying the effects of injection of Anisomycin on ITM or LTM any time window adequate for the training and testing of ITM and LTM could be used. An

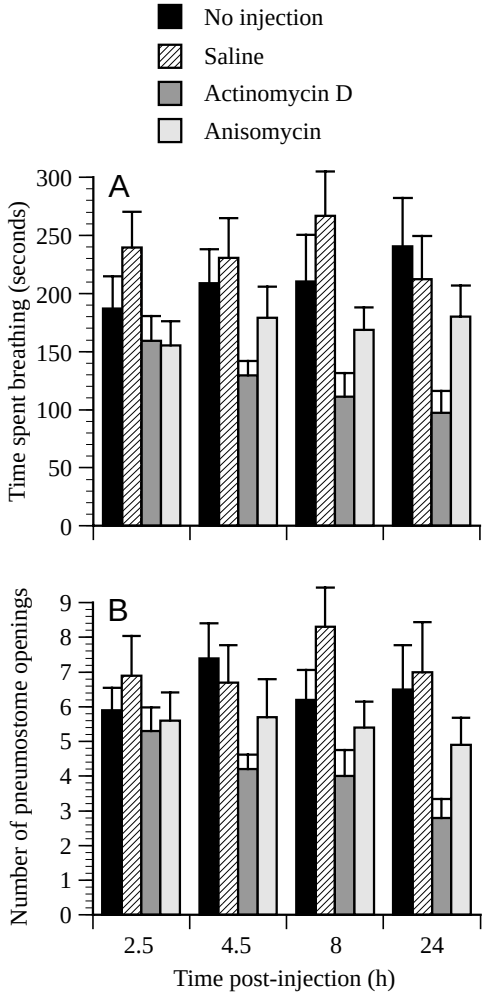

Fig. 1. The effect of protein synthesis blockers on transcription (Actinomycin D) and translation (Anisomycin) on spontaneous aerial respiratory behaviour in *Lymnaea*. (A) Breathing time observed during a 30 min session in hypoxic pondwater, at 2.5, 4.5, 8 and 24 h post-injection in (1) non-injected controls, (2) saline-injected controls, (3) Actinomycin D- and (4) Anisomycin-injected *Lymnaea*. All injections were into the foot of the snail. (B) Number of pneumostome openings observed using the above protocol. (N=20-25; see text).

injection time of 2.5 h prior to the operant conditioning training session was thus set, allowing enough time for diffusion of the drug within the snail.

By contrast, we found that injection of the transcription blocker Actinomycin D significantly altered aerial respiratory behaviour of *Lymnaea* beyond 8 h post-injection. There was no significant effect of Actinomycin D on the measured parameters of aerial respiratory behaviour (total breathing time and number of breaths) at 2.5 and 4.5 h post injection (ANOVA, $F_{2,21}$=2.0797; P=0.1376 and $F_{2,21}$=1.5849; P=0.217, respectively), nor were any significant differences in the measured respiratory parameters between the non-injected and Actinomycin D groups in the 2.5 h session found (two-sample t-test, P>0.05). There was, however, a significant decrease in both total breathing time and the number of openings in the Actinomycin D group between the first (2.5 h post injection) session and the fourth session (24 h post-injection) (Fisher's LSD protected t-test, P<0.05 and P<0.01; respectively). When we compared the total breathing time in session 3 (the 8 h session) with that of session 1, we obtained a P value of 0.053 (i.e. close to being significant) in a Fisher's LSD protected t-test. Since it was possible that the drug was interfering with the snails' breathing behaviour at periods longer than 8 h post-injection we limited our experiments to 8 h post injection.

Intermediate term memory

A single 30 min training session is sufficient to establish a memory that persists for 3 but not 4 h (Fig. 2). Two naïve cohorts of 20 snails each received an injection of saline 2.5 h before training as described in Materials and methods. The first cohort was tested for memory 3 h after the training session and the second 4 h after the training session. Memory was present for 3 h (Fig. 2A). That is, the number of attempted pneumostome openings in the memory test session was significantly fewer than in the training session (P<0.01), meeting the criterion for memory retention. However, when the second cohort was tested for memory 4 h after the training session, memory was not present (Fig. 2B). That is, the number of attempted pneumostome openings in the 30 min memory test session was not significantly different from the number in the training session (P>0.05). The criterion for memory retention was thus not met. We therefore conclude that a single 30 min training session is sufficient for memory retention of 3 h but not 4 h.

Knowing that we were able to produce an ITM that persisted for 3 h, we could test the effects of Actinomycin D and Anisomycin on other naïve cohorts of snails. We first tested the effect of the transcription blocker Actinomycin D. 20 snails were pre-injected with Actinomycin D 2.5 h before the training session (Fig. 3A). These injected snails performed similarly to the control snails. That is, the number of attempted pneumostome openings in the 30 min training session was similar (i.e. not statistically different; P>0.05) to the number we observed in Fig. 2. When we tested for memory in these Actinomycin D-injected snails we found that memory was present. That is, the number of attempted pneumostome openings in the memory test session was significantly less (P<0.01) than the number of attempted openings in the training session. To show that the statistically lower number of attempted openings in the memory test session was due to memory retention and not a side effect of the drug, we challenged these snails with the carrot-odour context 1 h later. As can be seen (Fig. 3A) the number of attempted openings in the carrot context session was significantly different from the number in the memory test session (P<0.01) but was not different from the number in the initial training session (P>0.05). We thus conclude that Actinomycin D does not prevent the formation of ITM.

We next performed a similar experiment using the

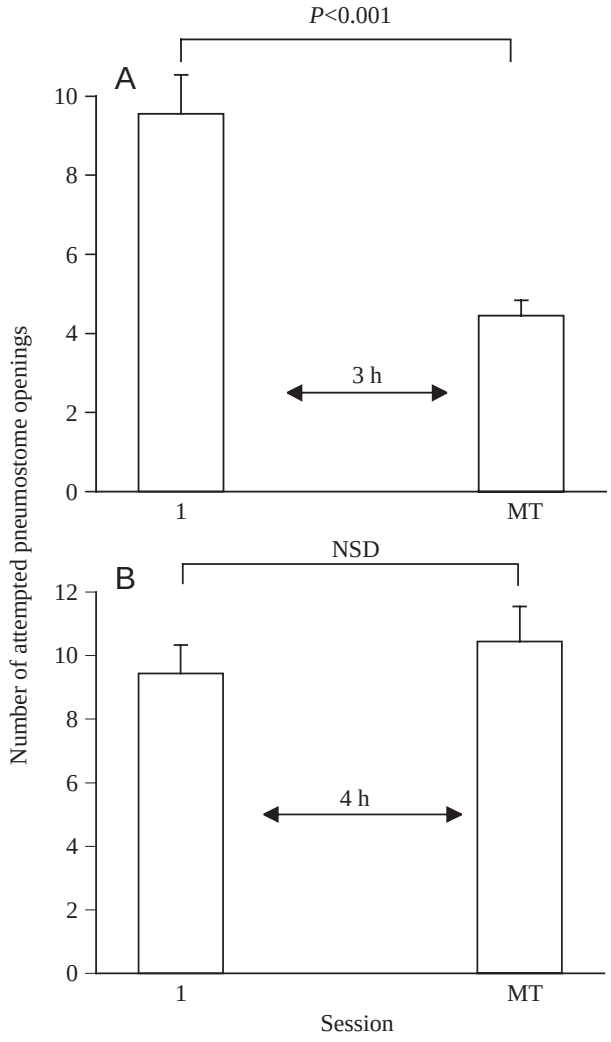

Fig. 2. A single 0.5 h training session is sufficient to produce a memory that persists for 3 h but not 4 h in *Lymnaea*. (A) 20 naïve snails were pre-injected with saline 2.5 h before receiving a single session of operant conditioning training of 0.5 h duration (1). When tested 3 h later (MT) memory was observed. That is, the number of attempted pneumostome openings in the memory test session was significantly less (P<0.01) than the number in the operant conditioning training session. (B) A second naïve cohort of snails (N=20) was trained as in A, except that memory was tested 4 h after the training session. No memory was observed. That is, the number of attempted openings in the memory test (0.5 h) session was not statistically different (NSD) from the number of attempted openings in the training session (P>0.05).

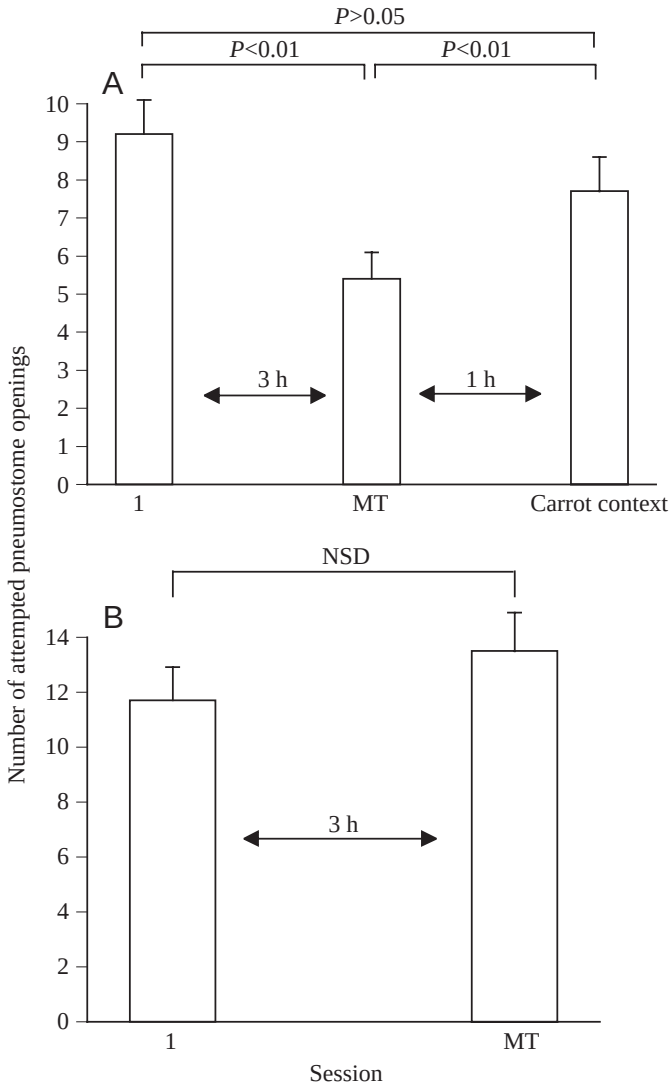

Fig. 3. The differential effect of a transcription and a translation blocker on ITM formation in *Lymnaea*. (A) A naïve group of snails (N=20) was injected with the transcriptional protein synthesis inhibitor Actinomycin D 2.5 h before the training session. Following the 0.5 h training session (1) the snails were tested for memory 3 h later (MT). Memory was present. That is, the number of attempted pneumostome openings in the memory test session was significantly less than the number of attempted openings in the operant conditioning training session (P<0.01). To show that this was a case of memory and not a drug-induced side effect, we altered the context 1 h later. When challenged with the 'carrot context' (see text) the snails attempted as many pneumostome openings as they did in the training session (P>0.05) and statistically more than in the memory test session (P<0.01). (B) As in A, except that snails were injected with the translational protein synthesis blocker Anisomycin. No ITM was observed when tested 3 h after the training session. That is, the number of attempted pneumostome openings in the memory test session was not statistically different (NSD) from the number in the training session (P>0.05).

translation blocker Anisomycin. Again a naïve cohort of snails (N=20) was pre-injected with Anisomycin 2.5 h before the initiation of training (Fig. 3B). The number of attempted pneumostome openings in the training session was not different from the number observed in the control snails (Fig. 2) or in the Actinomycin D group (Fig. 3A; P>0.05 in both cases). When we tested for memory retention 3 h later, however, we found that memory was not present. That is, the number of attempted pneumostome openings was not significantly different from the number in the single training session (P>0.05). Since these snails performed as they did in the initial training session we did not challenge them with the

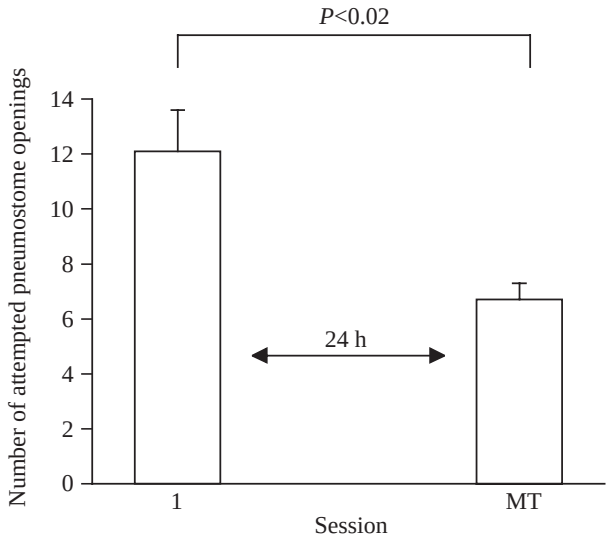

Fig. 4. A 1 h training session is sufficient to produce a memory in *Lymnaea* that persists for at least 24 h. A naïve cohort (N=20) of snails was injected with saline 2.5 h before operant conditioning training of 1 h duration (1). When tested for memory 24 h later (MT), memory was observed. That is, the number of attempted pneumostome openings in the memory test session was significantly less than the number of openings in the training session (P<0.01).

carrot-odour context. We conclude from this experiment that the translation blocker Anisomycin prevents the formation of ITM and, while protein synthesis is necessary for ITM, altered gene activity is not.

Long term memory

A single 1 h training session is sufficient to establish a memory that persists for 24 h (Fig. 4). A naïve cohort of 20 snails was pre-injected with saline 2.5 h before training commenced and 24 h later was tested for memory retention in a 1 h test. As can be seen, memory was present for at least 24 h. That is, the number of attempted pneumostome openings in the memory test session was significantly fewer than in the training session (P<0.01), meeting the criterion for memory retention. These data confirm our earlier reported results (Lukowiak et al., 2000) and show that we can study the effects of protein synthesis inhibitors on the establishment of LTM using a single 1 h training session.

We first tested the effect of the transcription blocker Actinomycin D on the LTM consolidation process by injecting a naïve cohort of snails (N=20; Fig. 5A) with Actinomycin D 2.5 h before the training session. Following the training session we tested whether the learning was consolidated into memory. As can be seen, memory was not observed as the number of attempted pneumostome openings in the 1 h memory test session was not significantly different from the number in the training session. Since these snails performed as they did in the initial training session we did not challenge them with the carrot-odour context. Notice also that the number of attempted pneumostome openings in the training session was not

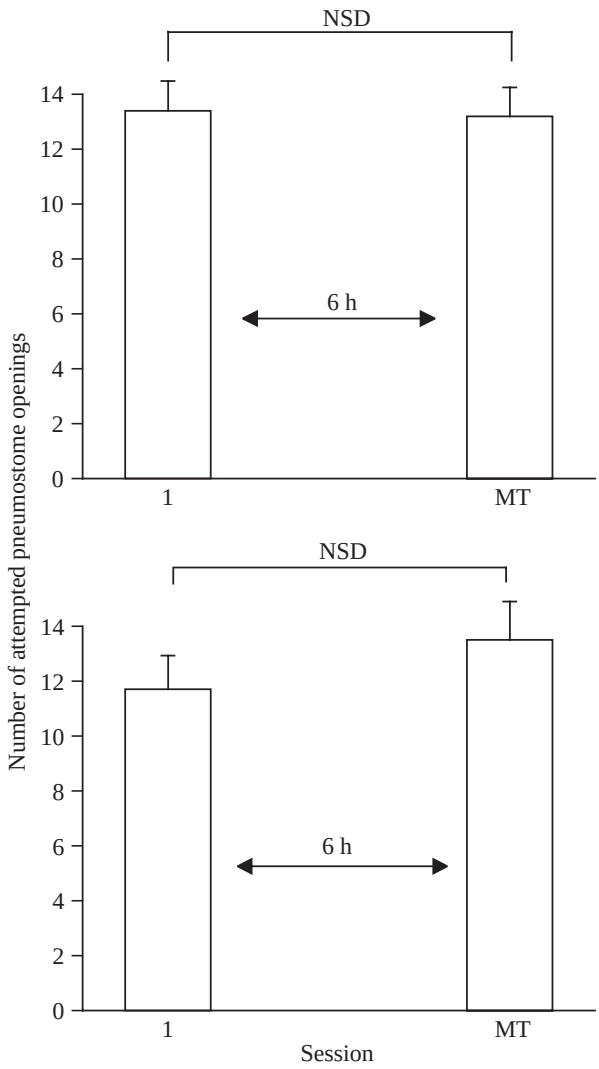

Fig. 5. Both transcription and the translation blockers prevent LTM formation in *Lymnaea*. (A) A naïve group of snails (N=20) was injected with the transcriptional protein synthesis inhibitor Actinomycin D 2.5 h before the 1 h training session (1). Following the 1 h training session the snails were tested for memory 6 h later (MT). Memory was not present. That is, the number of attempted pneumostome openings in the memory test session was not statistically different from the number in the training session (P>0.05). (B) As above, except the translational blocker Anisomycin was injected. Again, no memory was observed. That is, the number of attempted pneumostome openings in the memory test session was not statistically different from the number in the training session (P>0.05).

statistically different (P>0.05) from the number of attempted openings in the training session in the control snails in Fig. 4. Thus Actinomycin D, while not affecting the ability of the snails to perform aerial respiration, did block the establishment of memory.

In a similar manner we tested the ability of the translation blocker, Anisomycin, to prevent memory formation. Thus another cohort of naïve snails (N=20; Fig. 5B) was pre-injected

with the blocker 2.5 h before training commenced. As can be seen the learning was also not consolidated into memory. Thus, the number of attempted openings in the 1 h memory test session was not significantly different from the operant training session ($P>0.05$). Again the number of attempted openings in the operant training session in these Anisomycin pre-injected snails was not significantly different from the number in the LTM control (Fig. 4) or Actinomycin D groups (Fig. 5A; $P>0.05$). This demonstrates that Anisomycin did not alter the responsiveness of the snails but did alter their ability to consolidate the learning into LTM. Since in both the Actinomycin D and Anisomycin experiments the number of attempted openings in the memory test session was statistically as large as in the initial training session and in the LTM control group (Fig. 4), we did not have to alter the context to show that the snails were still responsive. We therefore conclude that LTM is dependent on both altered gene activity and new protein synthesis.

Discussion

We set out to determine if the processes underlying ITM and LTM formation in *Lymnaea*, subsequent to associative learning, were differentially susceptible to disruption by two different classes of protein synthesis blockers (transcription *versus* translation) and found that they are. We are now able to initiate further studies, using the data obtained here, to directly determine where and how long-lasting memory is encoded within the *Lymnaea* nervous system. Our working hypothesis is that both forms of memory are encoded within the three-neuron CPG that is essential for aerial respiratory behaviour.

Injection of the translation blocker Anisomycin into the hemocoel did not alter the responsiveness of the snails in either the ITM- or LTM-training procedure, but did prevent the consolidation of associative learning into both ITM and LTM. In our model system new protein synthesis is therefore a necessary step for encoding of both ITM and LTM. The transcription blocker Actinomycin D, in the time period used, also had no effect on the snails' responsiveness during training and, importantly, had no effect on the ITM formation, indicating that the transcription process is not a necessary step in ITM formation. The transcription process is necessary for encoding LTM, however, as Actinomycin D prevented its establishment. These findings are consistent with the hypotheses that the consolidation of learning into LTM is dependent on both altered gene activity and protein synthesis (Davis and Squire, 1984), whilst the transformation of learning into ITM is only dependent on protein synthesis (Rosenzweig et al., 1993; McGaugh, 2000; Sutton et al., 2001). A recent abstract, utilizing a one-trial associative learning procedure in *Lymnaea*, similarly concludes that LTM can be blocked by both translation and transcription blockers (Fulton et al., 2002).

The hypothesis that there are at least two stages in long-lasting memory formation, each dependent on different molecular mechanisms and subsequent cellular events, is supported by our data. With respect to ITM, our results are consistent with the hypothesis that the molecular message necessary for the induction and maintenance of ITM is already transcribed and only need be translated for ITM encodement. A requirement for protein synthesis but not mRNA synthesis in the intermediate phase of memory consolidation has previously been found in *Hermissenda* (Crow et al., 1999). Furthermore, the *Aplysia* sensory-motor neuron synapse undergoes a 5HT-induced facilitation termed ITF (intermediate term facilitation, lasting a few hours) that requires only translation (Ghirardi et al., 1995; Martin et al., 1997; Sutton et al., 2001). In addition, complementary data from three other studies are consistent with the hypothesis that the new proteins necessary for ITM are produced from pre-existing mRNA transcripts (Mauelshagen, 1998; Martin et al., 1997; Manseau et al., 1998). The synaptic ITF seen at the *Aplysia* sensory-motor neuron synapse may in part form the basis of a behavioural memory (ITM) that persists for a similar time course, and is similarly disrupted by translational but not transcriptional blockers, following tail-shock induced siphon-withdrawal sensitization in reduced preparations (Sutton et al., 2001).

Interestingly, evidence for protein translation outside of the soma has been emerging in both vertebrate and invertebrates (for a review, see Giuditta et al., 2002). For example, it has been demonstrated that the isolated primary neurite (i.e. with the soma removed) of a *Lymnaea* neuron has the capacity to synthesize *de novo* proteins from injected mRNA (van Minnen et al., 1997). New proteins translated from the injection of 'foreign' mRNA into the primary neurite of *Lymnaea* were functionally inserted into the membrane (Spencer et al., 2000). Similarly, in culture following the removal of the soma of a mechano-sensory neuron in *Aplysia*, the remaining primary neurite was capable of local translation of mRNAs (Martin et al., 1997). Since ITM is dependent on protein synthesis from a pre-existing message, it is possible that the protein synthesis responsible for ITM could occur in extrasomal regions at the site of plasticity (see below).

Experiments where the soma of RPeD1 in *Lymnaea* in the freely moving animal was ablated but the primary neurite left functionally intact before operant conditioning training, showed that the snails are still capable of performing aerial respiratory behaviour and are still able to learn and form ITM (Scheibenstock et al., 2002); however, they could not form LTM. We hypothesize that LTM formation did not occur because RPeD1, one of the CPG neurons, is a site of LTM formation. Since the RPeD1 nucleus was not present, the necessary transcription process for LTM could not occur. These snails were capable of forming ITM, so we further hypothesize that the necessary protein synthesis for ITM occurs extra-somally, possibly at the actual sites of synaptic plasticity, i.e. the pre- or post-synaptic specializations. Such locally synthesized proteins in snails where the soma is intact may serve to mark the site of plasticity so that new proteins being synthesized in the soma are delivered to specific sites (Martin et al., 1997; Manseau et al., 1998).

Why are there two different forms of longer-lasting memory, each with a different time course and susceptibility to interruption by different classes of protein synthesis blockers? Rosenzweig et al. (1993) put forward a scheme to explain the existence of ITM, with the central hypothesis that the underlying biochemical mechanisms (i.e. an assortment of classes of protein kinase activities and the difference in the requirement of transcription) responsible for each form of memory are not arranged in a serial fashion but rather occur in parallel. ITM forms sooner and persists for a shorter period of time than LTM, thus ITM may serve a function somewhat analogous to a memory cache of the CPU of a personal computer. According to this scheme, ITM allows a memory to be maintained until such time as LTM (which depends on events in the nucleus) can be induced, the newly synthesized proteins transported and becoming functional. The proteins subserving ITM could additionally serve as 'signposts' or 'markers' that ultimately enable the proteins encoded by the transcription process to arrive at the proper site of plasticity so that memory will become encoded only at specific sites and not globally within the neuron.

At the behavioural level it has been shown that prior ITM can augment the ensuing persistence of LTM (Smyth et al., 2002). In those experiments a training procedure that only produced a memory persisting for 3 h was shown to significantly augment the persistence of memory produced by an LTM-training procedure. This augmentation occurred up to 5 h after the last ITM training session, even though there was no behavioural evidence that ITM was present. Thus while the biochemical and molecular processes at the neuronal level that encode ITM and LTM may occur in a parallel, non-sequential, fashion it appears that there is an augmenting effect of the 'ITM-process' on the 'LTM-process'. It has been suggested that LTM formation is in itself a two-step process, whereby the first step parallels ITM formation and involves only protein synthesis, while the second step requires transcription to produce new products capable of mediating the physiological and morphological synaptic changes characterized by LTM (Feudenthal-Ramiro, 2000).

It is not clear why the processes that encode ITM only persist for a few hours. One hypothesis is that the new proteins initiated by the ITM-training, which are responsible for the encodement of the memory, 'fall below' some threshold level and evidence of memory can no longer be demonstrated behaviourally. Support for this comes from the ITM sensitization seen in *Aplysia* reduced preparations, which is dependent on continued cAMP-dependent protein kinase (PKA) activation (Sutton et al., 2001). As PKA activation decreases with time the translation of mRNAs ceases and the changes induced by local protein synthesis fall below some lower limit, resulting in a synapse that is no longer facilitated. In the case of the tail-shock induced siphon-withdrawal response sensitization seen in *Aplysia* (Sutton et al., 2001) the fall in PKA-induced local protein synthesis appears to happen quite quickly, within 90 min, whilst in *Lymnaea* the fall in local protein synthesis persists for at least 3 h. In the tail-shock induced sensitization in *Aplysia* and in other preparations (including honeybee, rodents and humans; Kamin, 1957; Menzel, 1983; Sutton et al., 2001) there is often a U-shaped graph of memory retention. That is, memory is strong initially, fades after some period, and then regains its strength. In the case of tail-shock induced sensitization of the siphon-withdrawal response in *Aplysia*, this means that if a memory test is administered after ITM is over (90 min or so), but before LTM becomes apparent (some 15+ h after the last tail shock) there is no evidence of memory. We have not yet seen such a phenomenon in our *Lymnaea* operant conditioning studies. However, we do know (Smyth et al., 2002) that even though ITM may not be apparent behaviourally after 3 h, its underlying neuronal substrates are able to exert an augmenting influence of subsequent LTM formation.

The data presented here are the first to our knowledge in *Lymnaea* where the dependence of ITM and LTM on protein synthesis has been demonstrated. These new findings further strengthen the use of *Lymnaea* as model system (Benjamin et al., 2000) in studies to investigate the causal neuronal mechanisms underlying associative learning and its various forms of memory.

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