The Journal of Experimental Biology 205, 131–140 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3761

Gone but not forgotten: the lingering effects of intermediate-term memory on the persistence of long-term memory

Kim Smyth, Susan Sangha and Ken Lukowiak*

Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

*e-mail: lukowiak@calgary.ca

Accepted 26 October 2001

Summary

Aerial respiratory behaviour can be operantly conditioned in *Lymnaea stagnalis* and, depending on the interval between the training sessions, memories of significantly different durations are produced. In naïve snails, a 15 min training procedure with a 30 min interval between three training sessions results in memory that persists for only 3 h (intermediate-term memory, ITM); whilst if the three 15 min training sessions are separated by a 1 h interval memory persists for 48 h (long-term memory, LTM). We found that if ITM training preceded LTM training, then LTM would persist for 24 h longer. This augmenting effect on LTM persistence could be demonstrated for up to 5 h following the last ITM training session, even though ITM was not observed at that time. However, if LTM training ensued 8 h after the last ITM training session, an augmented LTM did not occur. Extinguishing the memory produced by the ITM training procedure also prevented augmentation of LTM. That is, if an extinction procedure was given to the snails after the ITM training procedure, LTM did not persist longer than 48 h. Thus, at the behavioural level, ITM and LTM are interconnected.

Key words: snail, *Lymnaea stagnalis*, memory, training, behaviour, learning.

Introduction

The abilities to learn and remember are essential for the survival of all organisms (Squire and Kandel, 1999). A better understanding of the cellular mechanisms underlying memory formation and its storage are of paramount importance to the development of effective treatments and cures for memory-defective neurodegenerative diseases such as Alzheimer's disease (Mayford and Kandel, 1999; Milner et al., 1998). Researchers are only now beginning to unravel the cellular, biochemical and molecular differences underlying the different facets of memory and their different behavioural phenotypes (i.e. the persistence of memory) (Squire and Kandel, 1999; Kandel and Pittenger, 1999). To date, most recent studies on memory formation and its maintenance have concentrated on neural analogues of short-term memory (STM, lasting only a few minutes) and long-term memory (LTM) (Lechner et al., 1999; Martin et al., 2000). At the behavioural level, far less attention has been paid to a shorter-lasting form of LTM, which was termed intermediate-term memory (ITM, lasting a few hours) (Rosenzweig et al., 1993). Moreover, whether these forms of memory occur in a sequential or parallel fashion is also not clear; although data in support of the three forms of memory occurring in parallel are compelling (Emptage and Carew, 1993; Botzer et al., 1998; Izquierdo et al., 2000).

It has been known for some time that both transcription and translation are necessary for the formation of LTM (Davis and Squire, 1984; McGaugh, 2000). Inhibition of protein synthesis does not affect STM and will only disrupt LTM if it occurs within a critical time period (i.e. the consolidation period) following learning (Bourtchouladze et al., 1998). Studies using the marine gastropod *Aplysia californica* and the insect *Drosophila melanogaster* have revealed that LTM formation involves a cAMP-dependent mitogen-activated protein (MAP) kinase signal transduction cascade culminating in the activation of the cAMP response element binding protein (CREB) transcription factors (Tully, 1998; Mayford and Kandel, 1999; Silva et al., 1998). There appears to be evolutionary conservation of the molecular mechanisms underlying the LTM process such that similar processes occur in animals as diverse as snails and mammals (Mayford and Kandel, 1999; Silva et al., 1998; 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 from those required for LTM (Rosenzweig et al., 1993), it was widely believed that ITM was indistinguishable from LTM. Intermediate forms of memory have since been demonstrated on a behavioural level through classical conditioning of honeybees (Gerber et al., 1998) and of *Aplysia californica* feeding behaviour (Botzer et al., 1998) and through operant conditioning of aerial respiration in *Lymnaea stagnalis*

(Lukowiak et al., 2000). At the neuronal level, analogues of ITM have been demonstrated at *Aplysia californica* and *Hermissenda crassicornis* central nervous system synapses (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.

In *Lymnaea stagnalis*, intermediate-term (persisting for only 3 h) and long-term (lasting more than 18 h) memories can be differentially produced by modifying the interval between training sessions, the training session duration and the number of training sessions per day (Lukowiak et al., 2000). Preliminary data further show in *Lymnaea stagnalis* that LTM can be blocked by both transcriptional and translational blockers, whist ITM is blocked only by translational blockers (Sangha et al., 2001). A major advantage of conditioning aerial respiratory behaviour in *Lymnaea stagnalis* is that the neural circuitry controlling this behaviour is well established. Aerial respiration is controlled by a three-neuron central pattern generator (CPG) whose sufficiency and necessity have been demonstrated (Syed et al., 1990, 1992). Moreover, neural correlates of operant conditioning have been demonstrated in the CPG neurons in both isolated ganglia and semi-intact preparations (Spencer et al., 1999) (G. Spencer, M. Kazmi, N. Syed and K. Lukowiak, in preparation). This characterization and development of the *in vitro* CPG system governing aerial respiration in *Lymnaea stagnalis* has set the foundation for the future study of the cellular and molecular changes that constitute the various forms of memory.

Materials and methods

Laboratory-raised freshwater pond-snails, *Lymnaea stagnalis* (L.), maintained in aerated aquaria at room temperature (20–22 °C) in the snail facility at the University of Calgary, fed *ad libitum* on lettuce and with shell lengths of 22.5–25 mm, were used for all experiments.

Operant conditioning

All snails were trained using the basic operant conditioning procedure (Lukowiak et al., 1996, 1998, 2000). Briefly, animals were labelled with a permanent marker and then placed into 500 ml of hypoxic pond water in a 1000 ml beaker. Hypoxia significantly increases aerial respiratory drive (Lukowiak et al., 1996). The pond water was made hypoxic by bubbling N_2 through it for 20 min prior to placing the animals in the beaker. A 10 min acclimation period was given to the snails following their placement into the hypoxic pond water. During this period, they could perform aerial respiration. At the beginning of each 15 min operant conditioning training session, the animals were gently pushed under the surface of the water. During the operant conditioning training session, every time an animal opened its pneumostome, it was ‘poked’ in the pneumostome area with a hand-held sharpened wooden applicator. The tactile stimulus to the pneumostome area induced immediate closure of the respiratory orifice. The stimulus did not cause the snail to withdraw into its shell, and most animals stayed at the water surface following the stimulus. The time of each stimulus was recorded for every animal during the course of each session. Between training sessions, animals were returned to eumoxic pond water, where they could perform aerial respiration *ad libitum*.

ITM and LTM training procedures

To produce ITM, snails were subjected to a training protocol consisting of three 15 min training sessions, with each training session separated by a 30 min interval. In the LTM training procedure, the snails received three 15 min training sessions separated by a 1 h rest interval.

In the experiments designed to determine whether LTM persists for longer in snails given previous ITM training, we used the following procedure: snails received the ITM training procedure and after various (3, 4, 5, 8 or 24 h) periods in their home aquaria received the LTM training procedure. Memory tests were conducted 48 or 72 h after the final LTM training session.

Criteria for learning and memory

Learning and memory were operationally defined as in previous experiments (Lukowiak et al., 1996, 2000; Spencer et al., 1999). Learning is defined as a significant effect of training on the number of attempted pneumostome openings [one-way analysis of variance (ANOVA), $P<0.05$; followed by a *post-hoc* Fisher’s LSD protected *t*-test, $P<0.05$ for each separate session]. For learning to have occurred, the number of attempted pneumostome openings in the final training session had to be significantly less than the number of attempted pneumostome openings in the first training session.

Memory is present if: (i) the number of attempted pneumostome openings in the memory test session is not significantly different from the number of attempted openings in the last training session and (ii) the number of attempted openings in the memory test session is significantly less than the number of attempted openings in session 1. The memory test for the ITM training procedure was performed 3, 4, 5, 8 or 24 h after the last ITM training session, whilst the memory test for the LTM training procedure was performed 24, 48 or 72 h after the last LTM training session.

Extinction

Extinction was achieved by placing ITM-trained snails in the same hypoxic environment for 1.5 h. However, they were now allowed to breathe freely through their pneumostome. That is, no reinforcing stimuli were applied. Following ‘extinction training’, all snails were immediately trained for LTM as described above. Memory tests were conducted 48 or 72 h after the third LTM training session.

Yoked controls

In these experiments, animals (see Fig. 5) received a tactile stimulus to their pneumostome area not when they opened their

pneumostome, but when the snail to which they were yoked did. Thus, there was no contingency between the snail opening its pneumostome and the reinforcing stimulus. The ITM yoked control animals were given three yoked control training sessions using the data obtained from the snails given the ITM training procedure in Fig. 4. Following the third ITM yoked control session, these snails then received the LTM training procedure 3, 5, 8 and 24 h later. LTM was then tested 72 h later.

ITM-only training

Two different experiments were performed. In the first ITM-only control, a naïve cohort (N=15) of snails received six consecutive ITM training sessions. That is, each 15 min training session was separated by a 30 min interval. We then tested for LTM 72 h later.

In the second control experiment, a naïve cohort (N=20) of snails first received three ITM training sessions. Following a 4 h interval, these snails received a further three ITM training sessions. We tested for LTM 72 h later.

Results

ITM-only and LTM-only training

Naïve snails were subjected to either the ITM (two cohorts) or LTM (two cohorts) training procedures to demonstrate that learning and memory could be produced. The ITM procedure resulted in learning (Fig. 1), as did the LTM procedure (Fig. 2). Following the ITM training procedure, we found that memory was present when tested 3 h (Fig. 1A) but not 4 h (Fig. 1B) after the final ITM training session. Snails given the LTM training procedure showed memory when tested at 48 h (Fig. 2A) but not at 72 h (Fig. 2B) after the final LTM training session.

ITM followed by LTM training

We wanted to know whether the processes that encode ITM affect the processes that underlie LTM by either augmenting or decreasing memory persistence. Thus, non-naïve snails (i.e. those that had already received the ITM training procedure) were subsequently trained using the LTM training procedure 3, 4, 5, 8 or 24 h after the third ITM training session. All cohorts of these snails exhibited memory 48 h after the last LTM training session (in all cases, P<0.01 memory test session compared with session 6 and P>0.05 compared with session 1). As the data from all cohorts were similar, only the '3 h' and '4 h' cohorts are shown (Fig. 3). We picked these two cohorts because they demonstrate whether ITM is present or absent. Note that there is a difference in responsiveness on the first session (session 4) of LTM training between those two cohorts. This is due to the persistence of ITM in the '3 h' group. In the '3 h post-ITM training' cohort, the number of attempted pneumostome openings in the first LTM training session (session 4) was not significantly different from the third ITM training session (session 3; P>0.05), but both were significantly different from session 1 (P<0.01). In the other cohorts tested, the number of attempted pneumostome openings in session 4, the first LTM training session, was not significantly different from that in session 1 (P<0.01 in all cases) but was significantly different from that in session 3

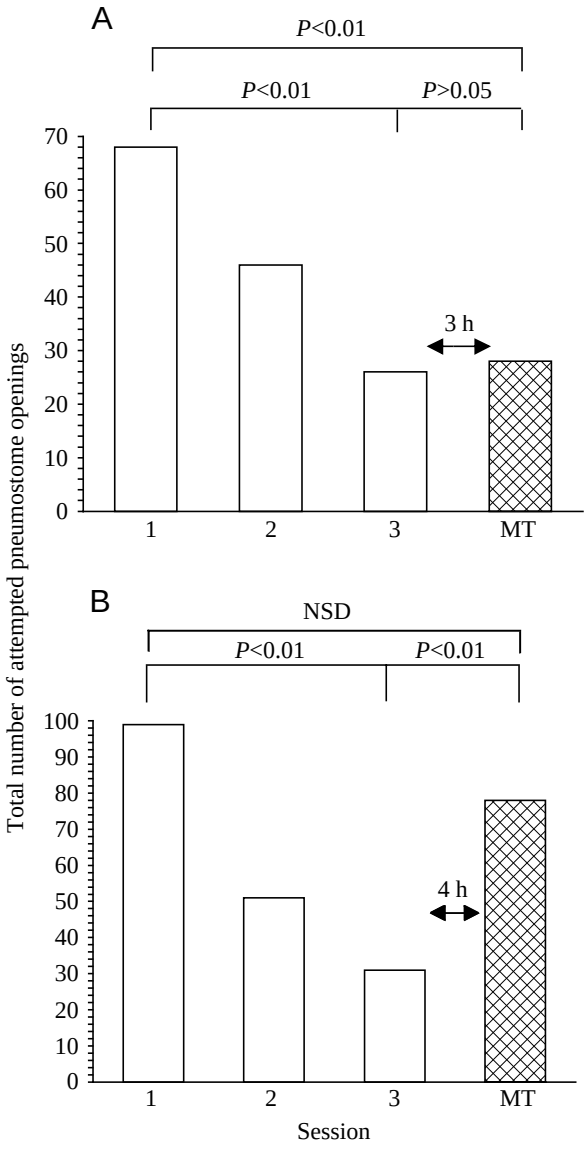

Fig. 1. The intermediate-term memory (ITM) training procedure results in learning and a memory that persists for 3 h but not for 4 h. (A) A cohort of 20 naïve snails received three 15 min operant conditioning training sessions, with each training session separated by a 30 min rest interval. Learning occurred (ANOVA, $F_{19,2}$=9.1613, P<0.001); session 3 was significantly different from session 1 (P<0.01). Memory was tested 3 h later (memory test, MT; cross-hatched column). There was no significant difference in the response between the MT and session 3 (NSD, P>0.05), but there was a significant difference between the response in session 1 and MT (P<0.01). (B) As in A, except that the MT was presented 4 h after session 3 (N=20). Learning occurred (ANOVA, $F_{19,2}$=15.7055, P<0.001); session 3 was significantly different from session 1 (P<0.01). There was no memory 4 h after the last training session. There was a significant difference between the response in session 3 and MT (P<0.01), but there was no significant difference between the response in session 1 and MT (NSD, P>0.05).

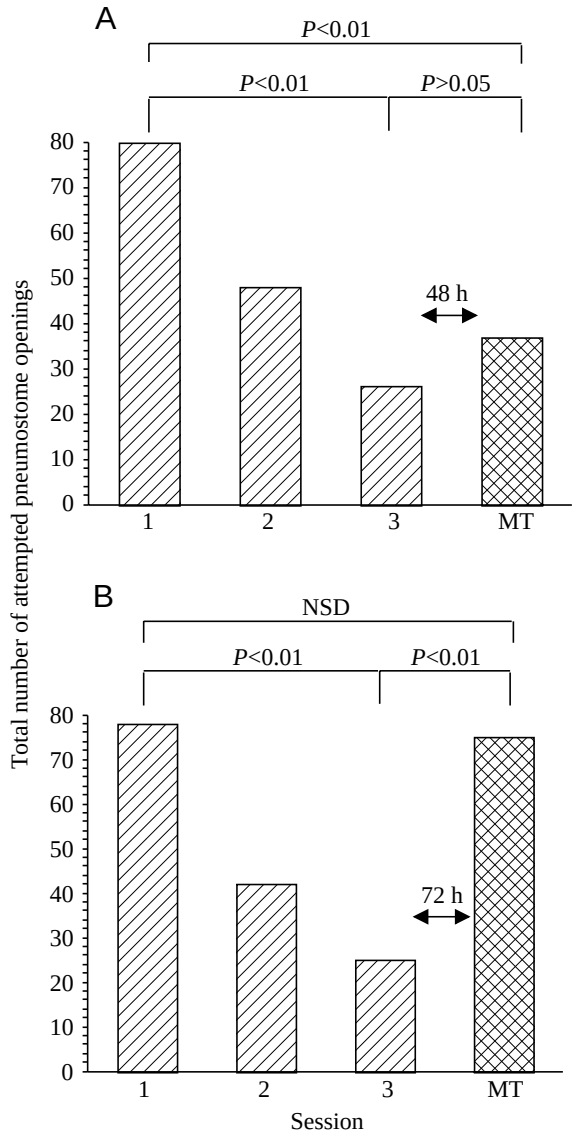

Fig. 2. The long-term memory (LTM) training procedure results in learning and memory that persists for 48 h but not for 72 h. (A) A cohort of 20 naïve snails received three 15 min operant conditioning training sessions, with each training session separated by a 1 h rest interval. Learning occurred (ANOVA, $F_{19,2}$=9.7738, P<0.001); session 3 was significantly different from session 1 (P<0.01). Memory was tested 48 h later (memory test, MT; cross-hatched column). There was no significant difference between the response in the MT and that in session 3 (NSD, P>0.05), but there was a significant difference between the response in session 1 and that in the MT (P<0.01). (B) As in A, except that the memory test (MT) was presented 72 h after session 3 (N=20). Learning occurred (ANOVA, $F_{19,2}$=11.4214, P<0.001); session 3 was significantly different from session 1 (P<0.01). There was no memory 72 h after the last training session. There was a significant difference between the response in session 3 and that in the MT (P<0.01), but there was no significant difference between the response in session 1 and that in the MT (NSD, P>0.05).

(P>0.05 in all cases), showing that the behavioural phenotype of ITM was no longer observable (e.g. the '4 h' cohort in Fig. 3B).

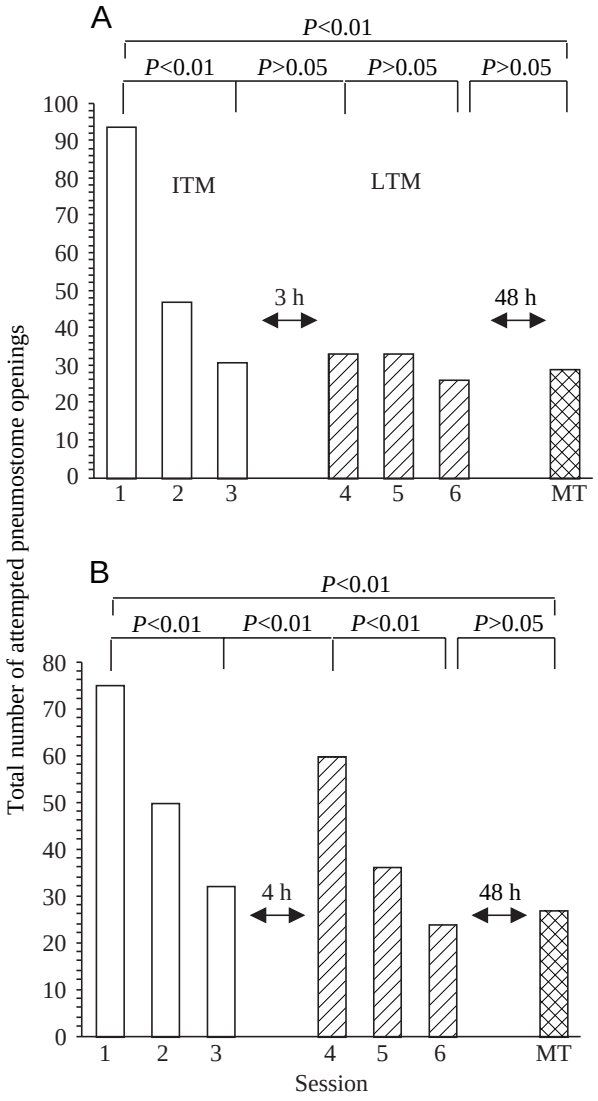

Fig. 3. Previous intermediate-term memory (ITM) training does not negatively affect the ability to form long-term memory (LTM). (A) A cohort of 20 naïve snails received three 15 min operant conditioning training sessions, with each training session separated by a 30 min rest interval. Learning occurred (ANOVA, $F_{19,2}$=12.1514, P<0.001); session 3 was significantly different from session 1 (P<0.01). Following a 3 h rest interval, these snails received the LTM training procedure, and memory was tested 48 h after the last LTM training session. The number of attempted pneumostome openings in session 4 was not significantly different (NSD, P>0.05) from that in session 3, indicating that ITM was present. The LTM training procedure resulted in no further statistically significant decrease in the number of attempted openings (session 4 was not significantly different from session 6, P>0.05). Memory was present when tested 48 h later because the memory test (MT) was not significantly different from session 6 (NSD, P>0.05), but was significantly different from session 1 (P<0.01). (B) As in A except that the LTM training procedure was initiated 4 h after the last ITM training session. Note that there was a significant difference between the response in session 3 and that in session 4 (P<0.01), indicating that there was no ITM. The previous ITM training did not interfere with the establishment of LTM at 48 h because MT was not significantly different from session 6 (NSD, P>0.05), but was significantly different from session 1 (P<0.01).

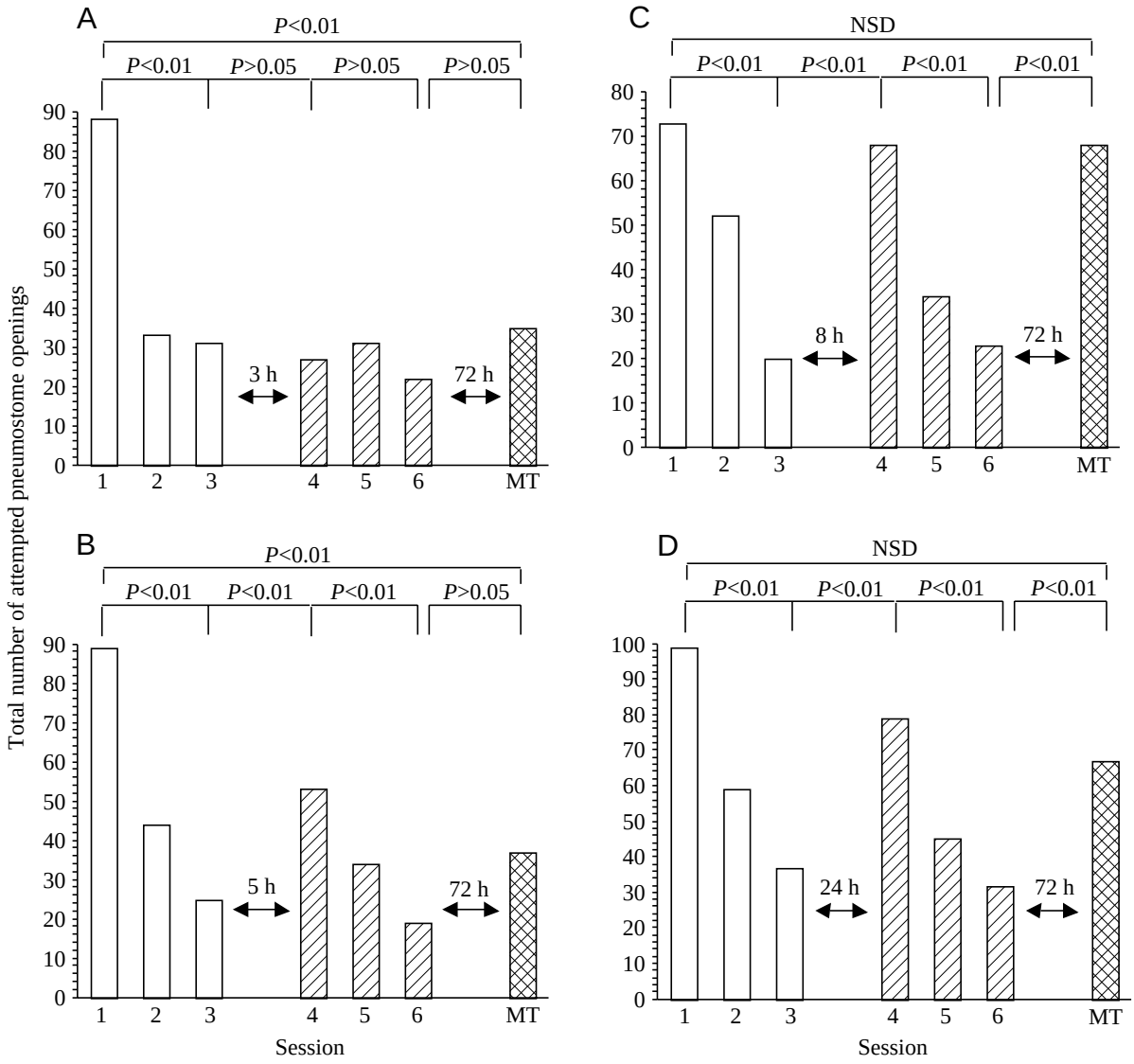

Fig. 4. Previous intermediate-term memory (ITM) training augments long-term memory (LTM) retention if LTM training occurs up to 5 h after the final ITM training session. A cohort of 20 naïve snails received ITM training and, as in Fig. 3A, exhibited memory at 3 h: the number of attempted pneumostome openings in session 4 was not significantly different (NSD, $P>0.05$) from that in session 3. Following this interval, these snails received the LTM training procedure and memory was tested 72 h after the last LTM training session. The LTM training procedure resulted in no further statistically significant decrease in the number of attempted openings: session 4 was not significantly different from session 6 ($P>0.05$). Memory was present when tested 72 h later because the memory test (MT) was not significantly different from session 6 (NSD, $P>0.05$), but was significantly different from session 1 ($P<0.01$). (B) As in A, except that the LTM training procedure was initiated 5 h after the last ITM training session. Note that there was a significant difference between the response in session 3 and that in session 4 ($P<0.01$), indicating that there was no ITM. Memory was present when tested 72 h later because MT was not significantly different from session 6 (NSD, $P>0.05$), but was significantly different from session 1 ($P<0.01$). (C) As in A, except that the LTM training procedure was initiated 8 h after the last ITM training session. In this group of snails, there was no augmentation of LTM. That is, there was a significant difference between session 6 and MT ($P<0.01$) but no significant difference between MT and session 1 (NSD, $P>0.05$), indicating no memory at 72 h. (D) As in C, except that the LTM training procedure was initiated 24 h after the last ITM training session. Again, there was no augmentation of LTM. There was a significant difference between session 6 and MT ($P<0.01$) but no significant difference between MT and session 1 (NSD, $P>0.05$), indicating no memory at 72 h.

We next asked whether LTM persisted longer in the '3 h post-ITM training' cohort than in the other cohorts. Naïve snails given the LTM training procedure (Fig. 2) have a memory that persists for 48 h but not for 72 h. When another '3 h post-ITM training' cohort was tested for memory retention, we found that LTM persisted for at least 72 h (Fig. 4A). That is, the previous ITM training resulted in a longer-lasting LTM. This led us to test both a '4 h post-ITM training' and a '5 h post-ITM training' cohort, even though ITM is not present behaviourally in these two groups. We were surprised to find that in these two cohorts LTM also persisted for 72 h (Fig. 4B; only the 5 h group is shown). Thus, in these

cohorts, the retention of LTM was prolonged. It therefore seemed logical to determine whether either an ‘8 h post-ITM training’ cohort or a ‘24 h post-ITM training’ cohort had a 72 h memory. In both these groups (Fig. 4C,D), we found that LTM did not persist for 72 h (just as it did in naïve snails).

Yoked control data

We have previously shown (Lukowiak et al., 1996, 2000; Spencer et al., 1999) that yoked control snails do not exhibit learning or memory. However, we needed to show that a yoked control procedure given to naïve cohorts of snails rather than the ITM training procedure did not result in the extension of LTM. These data are presented in Fig. 5. We performed yoked control training using the data sets for operant conditioning shown in Fig. 4. In none of the yoked control experiments did we observe an extension of memory and we therefore only present control data for the 3 h and 8 h intervals in Fig. 5. Two points are readily apparent. The first is that LTM is not present 72 h after the last (session 6) LTM training session. Thus, the presentation of ‘yoked’ tactile stimuli to the snails before LTM training does not extend the persistence of LTM. Second, note that there was no effect of the preceding yoked procedure on the number of attempted pneumostome openings in first session of LTM training (session 4) (compare these data with those presented in Fig. 4A, in which the preceding ITM training resulted in a memory that persisted for 3 h). These data show that it is the ITM training procedure that produces the extension of LTM and not just the presentation of non-contingent tactile stimuli to the pneumostome area.

Extinction and ITM controls

Given that previous ITM training could prolong the duration of LTM memory, we asked whether ‘extinction training’ between the ITM and LTM training sessions would suppress the augmentation of memory retention. Thus, we trained the snails for ITM, extinguished the ITM, trained the snails for LTM and finally tested for memory 48 or 72 h later.

The first group of snails (Fig. 6A) received a 1 h extinction training session following the third ITM training session. The interposition of the unreinforced training session was sufficient to extinguish ITM. That is, the number of attempted pneumostome openings in session 4 was significantly different from that in session 3 ($P<0.05$) but was not significantly different from that in session 1 ($P>0.05$). Subsequent to the extinction session, LTM training resulted in learning and in a memory that persisted for as long as it did in naïve snails (i.e. 48 h). In a second naïve cohort (Fig. 6B) subjected to the same ITM training, extinction and LTM training protocol, we tested for memory at 72 h and found that memory was not present. That is, the number of attempted pneumostome openings in the memory test session was significantly different from that in the last LTM training session (session 6; $P<0.01$) but was not significantly different from that in the first LTM training session (session 4; $P>0.05$). Similar data were obtained when ‘a 1 h extinction-training session’ was performed 2 or 3 h after the last ITM training session (data not shown). Collectively, these data show that, if ITM is actively extinguished prior to LTM training, there is no prolongation of LTM.

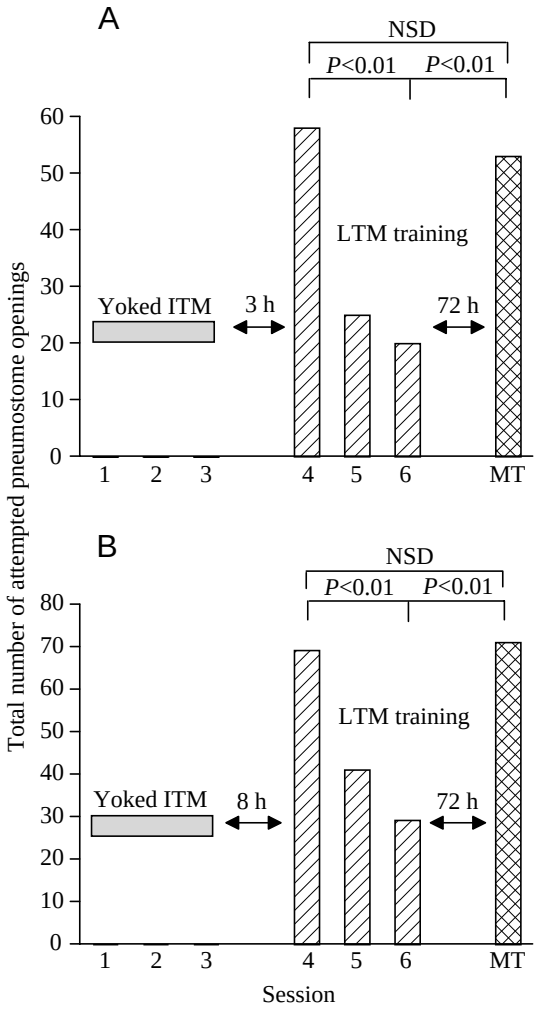

Fig. 5. Yoked controls do not show augmentation of long-term memory (LTM) retention. (A) A cohort of 15 naïve snails received three yoked control intermediate-term memory (ITM) training sessions. Three hours later, they received the LTM training procedure. Learning was evident (ANOVA, $F_{14,2}=15.2849$ $P<0.0001$; session 6 was significantly different from session 4, $P<0.01$), but memory was not present when tested 72 h after the last LTM training session: there was a significant difference between session 6 and the memory test (MT) ($P<0.01$) but no significant difference between MT and session 4 (NSD, $P>0.05$). (B) Another naïve cohort of snails ($N=15$) received three yoked control ITM training sessions. Eight hours later, they received the LTM training procedure. Learning was evident (ANOVA, $F_{14,2}=11.7037$ $P<0.0002$; session 6 was significantly different from session 4, $P<0.01$) but memory was not present when tested 72 h after the last LTM training session: there was a significant difference between session 6 and MT ($P<0.01$) but no significant difference between MT and session 4 (NSD, $P>0.05$).

Two final control experiments involving only ITM training sessions were performed. In the first of these control experiments, a naïve cohort of snails ($N=15$) received six ITM training sessions with a 30 min interval between each session

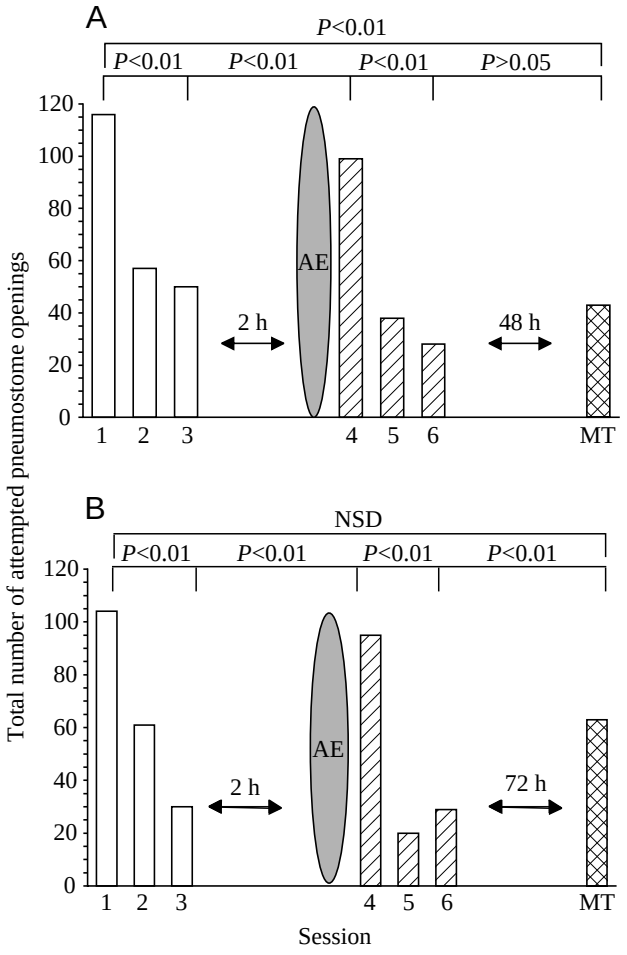

Fig. 6. Extinction training following intermediate-term memory (ITM) training prevents the augmentation of memory retention. (A) A cohort of 20 naïve snails received the ITM training procedure as in Fig. 1. Following a 2 h rest interval, these snails received extinction training (AE) before receiving the long-term memory (LTM) training procedure. Note that extinction training obliterated ITM. That is, the number of attempted pneumostome openings in session 4 was significantly different from that in session 3 (P<0.01) and was not significantly different from that in session 1 (P>0.05). The LTM training procedure resulted in a memory that persisted for 48 h. That is, the memory test (MT) was not significantly different from session 6 (NSD, P>0.05), but was significantly different from session 1 (P<0.01). (B) As in A, except that LTM was tested 72 h after the last LTM training session. Memory was not present because there was a significant difference between session 6 and MT (P<0.01) but no significant difference between MT and session 1 (NSD, P>0.05).

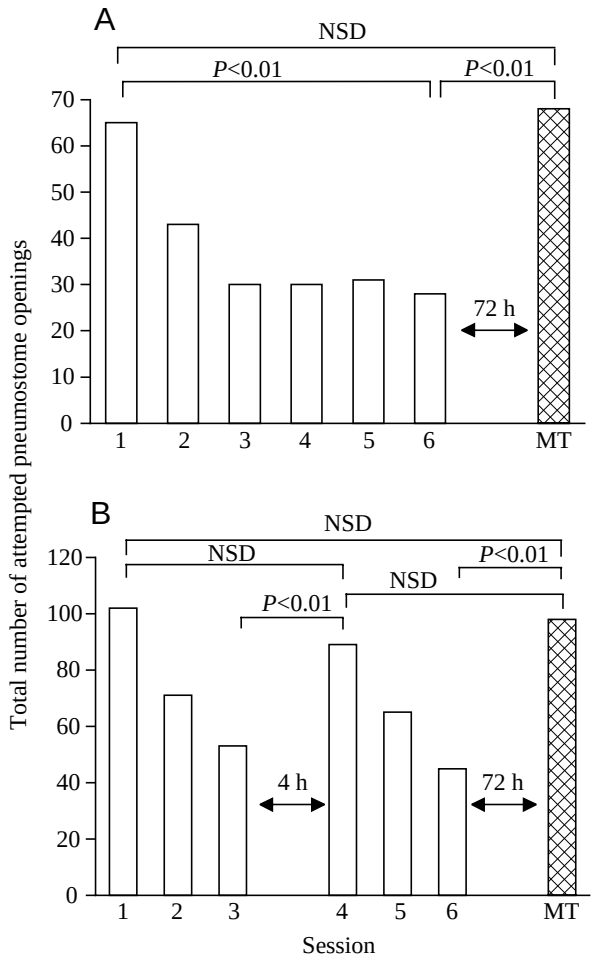

Fig. 7. Repeated intermediate-term memory (ITM) training by itself does not result in long-term memory (LTM). (A) A cohort of naïve snails (N=15) received six ITM training session (i.e. 15 min sessions separated by a 30 min rest interval). Learning was evident (ANOVA, $F_{14,5}$=14.2849, P<0.0001): session 6 was significantly different from session 1 (P<0.01); but memory was not present when tested 72 h later. That is, there was a significant difference between session 6 and the memory test (MT) (P<0.01) but no significant difference between MT and session 1 (NSD, P>0.05). (B) A naïve cohort of snails (N=20) first received three ITM training sessions and then, following a 4 h interval, received a further three ITM training sessions. In each sequence of ITM training, learning was observed (ANOVA, $F_{19,2}$=12.171, P<0.0001, in the first sequence; ANOVA, $F_{19,2}$=10.09, P<0.0003, in the second sequence). However, when LTM was tested 72 h after the last ITM training session (session 6), no memory was observed; i.e. MT was significantly different from session 6 (P<0.01) but was not significantly different from session 4 or session 1 (NSD, P>0.05 in both cases).

(Fig. 7A). This training procedure did not even result in a memory that persisted for 72 h. Thus, increasing the number of ITM training sessions (beyond three) does not result in LTM. In the second control experiment (Fig. 7B), a naïve cohort of snails (N=20) received three ITM training sessions and then, following a 4 h interval, they received three more ITM (rather than LTM) training sessions. We tested memory retention 72 h after the last ITM training session (session 6). As can be seen, there was no LTM.

Discussion

The purpose of these experiments was to explore the effects of previous ITM training on the persistence of memory following subsequent LTM training. That is, we were interested in determining whether the encodement of ITM has an effect on the persistence of LTM. We demonstrate here that, while the cellular processes underlying ITM and LTM may

occur in parallel and not sequentially (see below), they are interconnected at the behavioural level. We came to this conclusion by showing (i) that snails possessing an ITM made a longer-lasting LTM, (ii) that, even though the behavioural phenotype of ITM was not present, there was still a significant enhancing effect of previous ITM training on the establishment of longer lasting LTM, (iii) that extinction of ITM was possible and prevented the establishment of a longer-lasting LTM and (iv) that if, instead of ITM training, snails received a 'yoked procedure', no augmentation of LTM was observed.

There appear to be three facets of memory, characterized both by the length of time that the memory is present following the last training session and by their respective vulnerability to protein synthesis blockade. Various studies demonstrate that the underlying biochemical and molecular bases of the three facets of memory appear to be separate, distinct, parallel and not sequential (Milner et al., 1998; Martin et al., 2000; Mauelshagen et al., 1998; Manseau et al., 1998; Sutton et al., 2001).

It has been difficult to demonstrate behaviourally whether LTM could be formed without first eliciting ITM. In the rat, specific receptor antagonists given to different brain areas selectively block the expression of a memory persisting for 1.5 h without blocking a memory tested at 24 h (Izquierdo et al., 2000). However, it was not clear whether the ITM process had not been initiated and only its recall blocked. We took a different, 'positive' approach to the question by showing that previous training with a procedure that results only in ITM would potentiate LTM, as demonstrated by a longer-lasting memory. Thus, the processes that underlie ITM augment the establishment and/or maintenance of LTM. In addition, we found that ITM can enhance LTM even after behavioural ITM could not be demonstrated. That is, even though we could not detect ITM 4 h or 5 h after the third ITM training session, there was still a potentiating effect of the previous memory on the subsequent establishment and recall of LTM. However, this enhancing effect could not be demonstrated when the interval between the last ITM training session and the first LTM training session was greater than 5 h or when the ITM was extinguished prior to LTM training (see below). Thus, this as yet unidentified cellular process responsible for increasing LTM longevity does not persist indefinitely and can be rapidly made non-functional by extinction training.

Previously, in *Lymnaea stagnalis*, different training procedures have been shown to result in either ITM or LTM (Lukowiak et al., 2000). Here, we confirm these findings showing that a training period of 15 min is sufficient to produce either ITM or LTM, depending on the interval between training sessions. A 1 h interval between sessions is necessary for LTM formation, whilst a 30 min interval between sessions produces only ITM. Thus, the same amount of operant conditioning training results in significantly different memories, one persisting for 3 h and the other for 48 h. It is still not clear why a 30 min interval is not sufficient to produce the longer-lasting memory, but this inability may be due to the biochemical processes that are necessary to alter gene activity required for LTM (Carew, 1996; Crow et al., 1999) (see below).

It is not certain what molecular processes underlie the formation of ITM in *Lymnaea stagnalis* or, for that matter, in most other organisms. Because ITM requires new protein synthesis but not altered gene activity, translational but not transcriptional inhibitors block ITM formation (Crow et al., 1999; Sutton et al., 2001). Preliminary data obtained in *Lymnaea stagnalis* are in agreement with these previous data. Thus, anisomycin (an inhibitor of the translation process) blocks both ITM and LTM, whilst Actinomycin D (an inhibitor of the transcription process) blocks LTM but not ITM (Sangha et al., 2001). These findings are consistent with the hypothesis that the mRNA(s) necessary for ITM formation is already present at or near the sites where the memory is encoded. These sites can be extrasomal because *de novo* protein synthesis can occur outside the nucleus (Van Minnen et al., 1997; Martin et al., 1997; Spencer et al., 2000). ITM might therefore be a mechanism that 'marks' the site of memory encodement until such time as the new protein(s) made following the transcription process are delivered from the soma to form the longer-lasting LTM. Thus, it could be that, 4–6 h after the last ITM training session, there is still a threshold amount of the 'ITM protein' at the site of 'memory encodement' that allows for the more efficient delivery or insertion of the LTM protein. This would produce a more persistent LTM even though there is not sufficient 'ITM protein' available to produce the ITM behavioural phenotype. However, the 'ITM-evoked protein' is not sufficient by itself to encode LTM, as shown by the ITM-only (Fig. 7) experiments, in which no LTM was exhibited 72 h after the last ITM training session.

Consistent with the above notion are the data from extinction experiments. The interposition of extinction training following the ITM training resulted in no augmentation of memory persistence following LTM training. If extinction is viewed as a form of learning that co-exists with the previously learned behaviour and is initially more 'powerful' than the previously learned behaviour, then it is not surprising that there is no augmentation of the LTM-training-induced memory. The 'ITM protein' following extinction may have been either used up or replaced by the 'extinction protein'; thus, the site of 'memory encodement' would not be marked so that the ensuing LTM training does not result in a longer-lasting memory. Although extinction of LTM has already been demonstrated in *Lymnaea stagnalis* (McComb et al., 2001), extinction of ITM has not previously been demonstrated.

We also do not know the causes underlying the forgetting of ITM. A possibility is that, without further training, there is no signal to activate and/or maintain the 'ITM mRNA' to cause the local *de novo* protein synthesis necessary to maintain the memory. A second possibility is that there is only a limited amount of the 'ITM mRNA', which is used up by the initial ITM training procedure, and that the life of the protein is such that it can last only 3–4 h. Thus, memory is lost because the protein is lost or degraded to a subthreshold state that does not produce the behavioural phenotype of ITM.

How does the memory 'trace' work to increase memory persistence?

Our working hypothesis is that ITM training initiates translation of pre-existing mRNA into proteins capable of inducing the physiological and anatomical changes responsible for ITM. Some 3 h after the last ITM training session, these changes 'fall below' threshold level, and evidence of memory can no longer be demonstrated behaviourally. However, at the neuronal level, some of the changes persist longer (at least 5 h after conditioning). These changes constitute what we call the 'trace' of memory. This trace is sufficient to augment subsequent LTM formation and maintenance. It has been suggested that LTM formation is, in itself, a two-step process in which 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 (Freudenthal-Ramiro, 2000). It is possible that locally synthesized proteins (step 1) mark the site of plasticity so that new proteins being synthesized in the soma (step 2) are specifically delivered to target sites (Martin et al., 1997; Manseau et al., 1998). If the subsequent LTM training occurs after the trace has disappeared, then the neuron has to start rebuilding the memory from scratch. However, if the trace is still present during subsequent LTM conditioning, then the first phase of LTM formation will already be partially completed; additional changes can be reconstructed from a pre-formed framework. As a result, the LTM formed in the presence of the memory trace is built upon a stronger foundation, enabling it to last for longer.

It was suggested that if we blocked ITM formation with a translational blocker, such as anisomycin, we would see no extension of LTM. However, we have not been able to perform this experiment because we cannot 'wash out' the drug (the effects of anisomycin injection persist in the snail for 24 h). We are attempting to use a reversible protein translational blocker (4 °C cold-block) to perform these experiments. Our prediction is that blockage of protein synthesis with this translational blocker during the ITM training procedure will prevent any extension of memory produced by the subsequent (3–5 h later) LTM training procedure.

This work was supported by CIHR (K. L.).

References

Botzer, D., Markovich, S. and Susswein, A. (1998). Multiple memory processes following training that is a food inedible in *Aplysia*. *Learning Memory* **5**, 204–219.

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K. and Kandel, E. (1998). Different training procedures recruit one or two critical periods for context memory consolidation, each of which requires protein synthesis and PKA. *Learning Memory* **5**, 365–374.

Carew, T. J. (1996). Molecular enhancement of memory formation. *Neuron* **16**, 5–8.

Crow, T., Xue-Bain, J.-J. and Siddiqi, V. (1999). Protein synthesis-dependent and mRNA synthesis-independent intermediate phase of memory in *Hermissenda*. *J. Neurophysiol.* **82**, 495–500.

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

Emptage, N. and Carew, T. (1993). Long-term synaptic facilitation in the absence of short-term facilitation in *Aplysia* neurons. *Science* **262**, 253–256.

Freudenthal-Romiro, R. (2000). Participation of Re1/NF-kappaB transcription factors in long-term memory in the crab *Chasmagnathus*. *Brain Res.* **855**, 274–281.

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

Ghirardi, M., Montarolo, P. B. and Kandel, E. (1995). A novel intermediate stage in the transition between short- and long-term facilitation in the sensory to motor synapse of *Aplysia*. *Neuron* **14**, 413–420.

Hermitte, G., Pedreira, M., Tomsic, D. and Maldonada, H. (1999). Context shift and protein synthesis inhibition disrupt long-term habituation after spaced, but not massed, training in the crab *Chasmagnathus*. *Neurobiol. Learning Memory* **71**, 34–49.

Izquierdo, L., Vianna, M., Barros, D., Souza, T., Ardenghi, P., Sant, M., Rodrigues, C., Medina, J. and Izquierdo, I. (2000). Short- and long-term memory are differentially affected by metabolic inhibitors given into hippocampus and entorhinal cortex. *Neurobiol. Learning Memory* **73**, 141–149.

Kandel, E. R. and Pittenger, C. (1999). The past, the future and the biology of memory storage. *Phil. Trans. R. Soc. Lond. B* **354**, 2027–2052.

Lechner, H., Squire, L. and Byrne, J. (1999). One-hundred years of consolidation – remembering Muller and Pilzecker. *Learning Memory* **6**, 77–87.

Lukowiak, K., Adatia, N., Krygier, D. and Syed, N. I. (2000). Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. *Learning Memory* **7**, 140–150.

Lukowiak, K., Cotter, R., Westley, J., Ringseis, E., Spencer, G. and Syed, N. (1998). Long-term memory of an operantly conditioned respiratory behaviour in *Lymnaea stagnalis*. *J. Exp. Biol.* **201**, 877–882.

Lukowiak, K., Ringseis, E., Spencer, G., Wildering, W. and Syed, N. (1996). Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. *J. Exp. Biol.* **199**, 683–691.

Manseau, F., Sossin, W. and Castellucci, V. (1998). Long-term changes in excitability induced by protein kinase C activation in *Aplysia* sensory neurons. *J. Neurophysiol.* **79**, 1210–1218

Martin, K., Casadio, A., Zhu, H., Yaping, E., Rose, J., Chen, M., Bailey, C. and Kandel, E. (1997). Synapse-specific long-term facilitation of *Aplysia* sensory to motor synapses: A function for local protein synthesis in memory storage. *Cell* **91**, 927–938.

Martin, S. J., Grimwood, P. D. and Morris, R. G. M. (2000). Synaptic plasticity and memory: An evaluation of the hypothesis. *Annu. Rev. Neurosci.* **23**, 649–711.

Mauelshagen, J., Sherff, C. and Carew, T. (1998). Differential induction of long-term synaptic facilitation by spaced and massed applications of serotonin at sensory neuron synapses of *Aplysia californica*. *Learning Memory* **5**, 246–256.

Mayford, M. and Kandel, E. (1999). Genetic approaches to memory storage. *Trends Genet.* **15**, 463–470.

McComb, C., Sangha, S., Qadry, S., Yue, J., Scheibenstock, A. and Lukowiak, K. (2001). Context extinction and associative learning in *Lymnaea*. *Neurobiol. Learning Memory* (in press).

McGaugh, J. (2000). Memory, a century of consolidation. *Science* **287**, 248–251.

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

Rosenzweig, M. R., Bennett, E. L., Colombo, P. J. and Serrano, P. A. (1993). Short-term, intermediate-term and long-term memories. *Behav. Brain Res.* **57**, 193–198.

Sangha, S., Scheibenstock, A., Qadry, S. and Lukowiak, K. (2001). Extinction as a form of learning. In *Proceedings of the Learning Memory Conference*, p. 50. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Silva, A., Kogan, J., Frankland, P. and Kida, S. (1998). CREB and memory. *Annu. Rev. Neurosci.* **21**, 127–148.

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

Squire, L. and Kandel, E. (1999). *Memory: From Molecules to Mind.* Scientific American Library, New York.

Sutton, M., Mastyers, S., Bagnal, M. and Carew, T. (2001). Molecular

mechanisms underlying a unique intermediate phase of memory in *Aplysia*. *Neuron* **31**, 143–154.

Syed, N. I., Bulloch, A. G. M. and Lukowiak, K. (1990). *In vitro* reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea*. *Science* **250**, 282–285.

Syed, N., Ridgway, R., Lukowiak, K. and Bulloch, A. (1992). Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. *Neuron* **8**, 767–774.

Taubenfeld, S., Milekic, M., Monti, B. and Alberini, C. (2001). The consolidation of new but not reactivated memory requires hippocampal C/EBPB. *Nature Neurosci*. **4**, 813–818.

Tully, T. (1998). Toward a molecular biology of memory. The light's coming on. *Nature Neurosci.* **1**, 543–545.

Van Minnen, J., Bergman, E., Van Kesteren, R., Smit, A., Geraerts, W., Lukowiak, K., Hasan, S. and Syed, N. (1997). *De novo* protein synthesis in isolated axons. *Neuroscience* **80**, 1–7.