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# Perturbation of the activity of a single identified neuron affects long-term memory formation in a molluscan semi-intact preparation

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**Summary**

**The aim of this study was to investigate the neural basis of operant conditioning in a semi-intact preparation of the pond snail, *Lymnaea stagnalis*. *Lymnaea* learns, *via* operant conditioning, to reduce its aerial respiratory behaviour in response to an aversive tactile stimulus to its open pneumostome. Here we report the successful conditioning of naïve semi-intact preparations to show 'learning in the dish' and long-term memory that persists for at least 18 h. The neurons that generate this behaviour are readily identifiable and, for the first time, we have recorded from a neuron during a training paradigm that leads to long-term memory formation in the same preparation. Specifically, we recorded from the respiratory neuron Right Pedal Dorsal 1 (RPeD1), which is part of the respiratory central pattern generator and initiates the aerial respiratory behaviour. Previous studies have shown that long-term memory of this behaviour results in reduced RPeD1 activity. In the present study, we demonstrate that preventing RPeD1 impulse activity between training sessions reduces the number of sessions needed to produce long-term memory in our semi-intact preparation.**

Key words: operant conditioning, learning, long-term memory, semi-intact preparation, central pattern generator, mollusk, *Lymnaea stagnalis*.

## Introduction

One of the main challenges in the study of the neural basis of associative learning and memory is to relate the cellular changes directly to the observed changes in behaviour. One solution to this problem is in the use of a semi-intact preparation, which is dissected to expose the nervous system of the animal and allows simultaneous monitoring of neuronal activity and behaviour. It is well known that semi-intact preparations are capable of associative learning and memory (Horridge, 1962; Woollacott and Hoyle, 1977; Farley et al., 1983; Lukowiak and Colebrook, 1988; Cook and Carew, 1989; Crow and Forrester, 1991; Kemenes et al., 1997; Tsitolovsky and Shvedov, 1997; Antonov et al., 2001; McComb et al., 2005). However, a naïve semi-intact preparation capable of being operantly conditioned to show long-term memory (LTM) in the dissection dish has not previously been reported. The question therefore remains: how does one investigate, directly, the neural basis of associative learning in identified neurons during the formation of LTM? To address this question we have developed a semi-intact preparation of the pond snail *Lymnaea stagnalis* that is fully viable for up to 30 h. We have utilized this novel, long-lasting semi-intact preparation to investigate the neural basis of operant conditioning in *Lymnaea*. Operant conditioning is a form of associative learning that requires an association between an external stimulus and a behavioural response. *Lymnaea* performs aerial respiration *via* the opening of its primitive lung or pneumostome at the air–water interface (Jones, 1961). Previous studies have shown that this behaviour can be reduced *via* operant conditioning and that intact animals demonstrate both learning and LTM (Lukowiak et al., 1996; Lukowiak et al., 2000). Furthermore, operant conditioning of this behaviour has been shown to be context-dependent (Haney and Lukowiak, 2001) and the memory can be extinguished (McComb et al., 2002; Sangha et al., 2002; Sangha et al., 2003a).

Underlying the aerial respiratory behaviour of this animal is a well-characterized neural network or central pattern generator (CPG; Fig. 1A). The respiratory CPG comprises at least three neurons, Right Pedal Dorsal 1 (RPeD1), Input 3 Interneuron (IP3) and Visceral Dorsal 4 (VD4; Syed et al., 1990). RPeD1 receives excitatory chemosensory input from the periphery (Inoue et al., 2001) and its activity initiates and coordinates IP3 and VD4 activity, which in turn control pneumostome opening and closing respectively (Syed et al., 1990; Syed et al., 1991; Syed et al., 1992; Syed and Winlow, 1991). It has previously been shown that ablating the soma of RPeD1 prevents the formation of LTM following operant conditioning of the respiratory behaviour in intact animals (Scheibenstock et al., 2002). Furthermore, studies using either

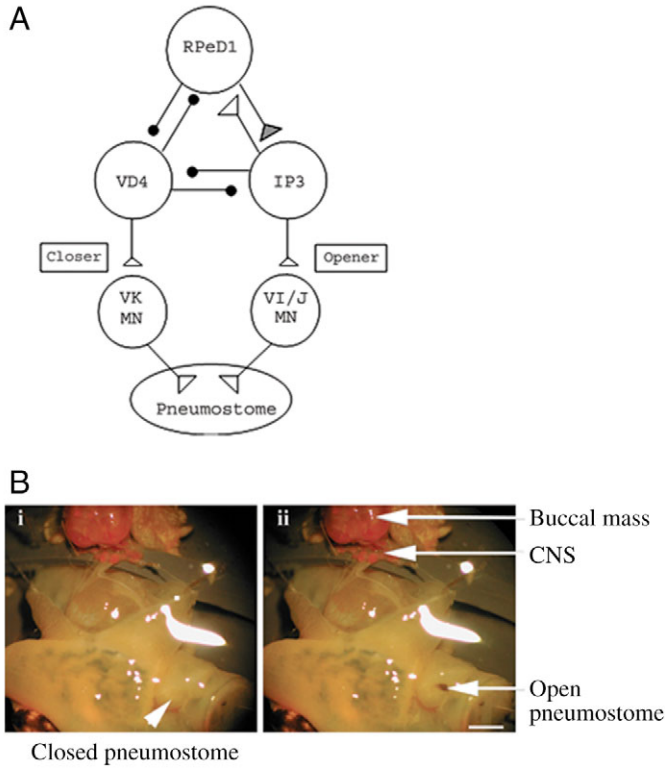


Fig. 1. Illustration of pneumostome opening and the underlying circuitry in *Lymnaea*. (A) Schematic diagram of the three neuron CPG and motorneurons innervating the pneumostome muscles. RPeD1, right pedal dorsal 1 neuron; VD4, visceral dorsal 4; IP3, input 3 interneuron; VK, visceral K closer motorneurons (MN); VI/J, visceral I/J opener motorneurons; filled circles, inhibitory connections; open triangles, excitatory connections; grey triangles, biphasic connections (excitation followed by inhibition). (B) An example of pneumostome opening in a typical semi-intact preparation. The pneumostome is located on the bottom right of each panel and is closed, but reared upward in (i) and fully open in (ii). The two photographs were taken in quick succession (1 s apart) after lowering the saline level to expose the pneumostome to the water surface. Scale bar, 3 mm.

isolated brains or semi-intact preparations dissected from previously trained animals, have shown that the spontaneous activity of RPeD1 is reduced following conditioning (Spencer et al., 1999; Spencer et al., 2002; McComb et al., 2005). These previous studies suggest that gene activity as well as the impulse activity in RPeD1 likely play an important role in learning and/or long-term memory formation in *Lymnaea*. The primary objective of this study was to use our novel long-lasting semi-intact preparation to investigate directly, the role of RPeD1 activity in LTM formation.

## Materials and methods

### *Specimens*

Laboratory bred specimens of *Lymnaea stagnalis* L. were kept in well-aerated pond water prior to use. Some animals were received as a gift from the University of Calgary and the Free University of Amsterdam. Their diet consisted of romaine lettuce and Spirulina (Tetraphyll; Brampton, ON, Canada) fish food. The shell size of all snails used in these experiments ranged from 25 to 30 mm in length (i.e. 3–6 months old).

### *Dissection of semi-intact preparations*

Previous semi-intact preparations of *Lymnaea* used in learning and memory studies have been viable only up to 2–3 h (McComb et al., 2003; McComb et al., 2005; Spencer et al., 2002), thus negating the possibility of operantly conditioning a naïve semi-intact preparation to form LTM. A possible reason for the short-lived viability of this semi-intact preparation may be related to its much reduced nature (i.e. much of the animal's body wall and foot around the CNS was removed). Syed et al. (Syed et al., 1991) previously used a more intact preparation of *Lymnaea* to gain access to the animal's CNS, to characterize the neural basis of aerial respiration. The longevity of this preparation for use in operant conditioning studies in *Lymnaea* has not yet been reported. We used a similar approach to Syed et al. (Syed et al., 1991) for the dissection of our semi-intact preparation. Snails were first anaesthetized in a *Lymnaea* saline solution (Winlow and Haydon, 1981) containing 30% Listerine (containing menthol, 0.042% w/v; Toronto, ON, Canada), for 3 min. Listerine is a standard anesthetic used in *Lymnaea* studies, and its application has been shown not to affect memory (Spencer et al., 2002). Following this, the outer shell of the snail was removed, and its body was pinned in a Sylgard dish filled with saline. A medial incision was made from the base of the snail's mantle to its head to expose the inner cavity containing the central ring ganglia. The reproductive organs and esophagus were then removed. Medial cuts were made beneath the central ring ganglia to make it possible to place a small piece of Sylgard underneath the pedal ganglia. The commissure linking the left and right cerebral ganglia was severed and pinned down onto the Sylgard supporting the ventral surface of the pedal ganglia. This procedure fully exposed the entire dorsal surface of the central ring ganglia (Fig. 1B). In all preparations, the outer sheath covering the right pedal ganglion was removed using fine forceps. Finally, the pneumostome was raised slightly using a piece of Sylgard that was strategically placed underneath a portion of the snail's mantle. Each preparation was given at least 30 min to recover from surgery prior to the first training session.

### *Operant conditioning of naïve semi-intact preparations*

Conditioned semi-intact preparations were trained using a similar paradigm to successfully condition intact snails for LTM, described previously (Lukowiak et al., 2000; Lowe and Spencer, 2002; Sangha et al., 2003a). Our training paradigm consisted of four 20 min training sessions each separated by a 1 h interval. The memory test was conducted 18 h after the fourth (final) training session. The duration of the memory test was 20 min. To increase the frequency of aerial respiration in our semi-intact preparations, the saline bathing the preparation was made hypoxic by gently bubbling through a 90% $N_2$/10% $O_2$ gas mixture for 10 min prior to commencing each training

session and memory test (Spencer et al., 2002). The preparation was kept hypoxic throughout the training sessions by continuing to disperse the 90% $N_2$ /10% $O_2$ gas over the surface of the saline (Spencer et al., 2002).

During the training sessions and memory test, semi-intact preparations in the conditioned group received a mild aversive tactile stimulus to their open pneumostome at the air–water interface each time an attempt was made to perform aerial respiration (Fig. 1Bii). This resulted in closure of the pneumostome. The aversive tactile stimulus was applied using a blunt plastic probe. The time points for the delivery of the aversive tactile stimulus to conditioned preparations were recorded throughout each 20 min training session/memory test. Semi-intact preparations in the yoked control group (i.e. a semi-intact preparation randomly paired with a conditioned semi-intact preparation) also received the exact same number of aversive tactile stimuli to the same area of the pneumostome (which was accessible whether the pneumostome was open or closed). For the yoked controls, the delivery of the aversive tactile stimulus was similar in strength and corresponded with the time points when its conditioned mate received contingent reinforcement. The aversive stimulus was thus delivered to the yoked controls whether its pneumostome was open or closed (that is, due to the time-locked stimuli, the yoked controls may have occasionally received a contingent stimulus). A third group, the naïve control semi-intact preparations, did not receive any tactile stimuli to their open pneumostome while performing aerial respiration under the same hypoxic conditions. All three test groups were monitored separately. At the end of each training session, fresh saline was added to the dish to completely re-immerse the preparation's pneumostome and mantle, thus preventing pneumostome opening between training sessions. This was also done to prevent the preparation from drying out between sessions. All semi-intact preparations were kept at room temperature (20–22°C) for the entire duration of each experiment. In a subset of experiments a separate group of conditioned and control preparations were given only two 20 min training sessions (separated by a 1 h interval and followed by a 20 min memory test 18 h later).

### *Data collection and statistical analysis*

Learning and LTM were assessed in yoked and conditioned preparations by conducting 'freely behaving' pre-and post-test sessions in both groups (McComb et al., 2005). That is, the total breathing time and number of openings was assessed in preparations permitted to behave freely in a 20 min pre-test session. The two groups then underwent either the yoked or conditioned stimulation paradigm (four sessions and memory test). This was then followed by a freely behaving post-test, where the total breathing time and number of openings were again recorded. This paradigm was used to validate the yoked control procedure and to confirm that only the conditioned preparations showed a reduction in behaviour.

For all further experiments, the average number of attempted pneumostome openings of the conditioned semi-intact preparations was monitored across all training sessions and memory test (Lukowiak et al., 1996; Lukowiak et al., 1998; Spencer et al., 1999; Spencer et al., 2002). Unlike the conditioned group, however, whose pneumostome openings were interrupted by the stimulus, the yoked and naïve control preparations were able to open their pneumostomes uninterrupted. Thus for all control preparations, both the number of pneumostome openings as well as the total breathing time during each session were monitored as determinants of any possible changes in respiratory behaviour.

Since the same semi-intact preparation was tested across the four training sessions and in the memory test for each experiment, all statistical analysis (unless otherwise stated) incorporated a repeated measures design. A two-way repeated measures analysis of variance (two-way RM-ANOVA) was carried out to test for a possible interaction effect between the two independent variables (i.e. the treatment groups and training sessions/memory test). All *post-hoc* analysis was carried out using a corrected Bonferroni *t*-test for planned paired comparisons. Due to the repeated measures design of our study, most *post-hoc* analyses focused on within-group differences across the training sessions/memory test. Results were considered significantly different if $P<0.05$ was achieved. All data analysis was carried out using GraphPad Prism (version 3.0, Graph Pad Software Inc., San Diego, CA, USA). In all figures, the error bars represent the standard error of the mean (s.e.m.).

### *Hypoxic stress challenge of naïve semi-intact preparations*

Hypoxic conditions were kept the same as for the operantly conditioned group, except that the saline level was lowered enough to expose the mantle for cutaneous gas exchange, but not enough to expose the pneumostome for aerial respiration. These control preparations were initially allowed to perform aerial respiration during a 20 min pre-observation period. They were then subjected to five 20 min hypoxic stress sessions to replace the four training sessions and memory test undergone by conditioned preparations. During these hypoxic stress sessions, the animals were prevented from performing aerial respiration. At the end of each hypoxic stress session the saline was replaced with fresh saline. The ability of these semi-intact preparations to perform aerial respiration was assessed following the last hypoxic stress session, in a 20 min post-observation session. The data obtained from the post-observation session were then compared to the pre-observation session.

### *Electrophysiological recordings*

Intracellular recordings from the CPG neuron, RPeD1, were performed between training sessions 1 and 2 (in the absence of pneumostome activity), using standard electrophysiological techniques. Glass microelectrodes (resistance 20–40 MΩ) were positioned using a Leitz ACS01 micromanipulator (Charolette, VT, USA). Cell penetration was aided by applying non-specific solid protease (Sigma Chemicals, St Louis, MO, USA; sigmatype XIV) for 3 min over the surface of the right pedal ganglion (in order to soften the inner sheath). The

protease treatment was terminated by rinsing the entire volume of the holding chamber three times with distilled water, which was then refilled with fresh saline. Electrophysiological signals were obtained using an intracellular electrometer (Warner Instruments, IE-210, Harden, CT, USA) connected to a Power Lab digital acquisition system, (model\4SP; AD Instruments, Charolotte, NC, USA) with Chart software (version 4.1). The impulse activity of RPeD1 was prevented by using the DC current source of the electrometer to pass hyperpolarizing current (0.4–1.4 nA) through the microelectrode in the soma. The DC power source of the electrometer was also used to steadily pass depolarizing current (0.4 nA) through the microelectrode to increase the frequency (Hz) of RPeD1's impulse activity. The resting membrane potential of RPeD1 was measured on penetration of the cell.

## Results

### *Learning and LTM in the semi-intact preparation*

Prior to surgery, each snail was randomly assigned to one of the three behavioural test groups: operantly conditioned, yoked control or naïve control. For the operantly conditioned group, the delivery of the aversive tactile stimulus was always contingent on the opening of the preparation's pneumostome (see Fig. 1Bii). The aversive tactile stimulus was not delivered if the pneumostome moved but did not open (Fig. 1Bi). All preparations were given four sessions separated by a 1 h interval. This was then followed by the memory test 18 h after the final session. It has previously been shown that 15 min training sessions (separated by 1 h) is sufficient to produce LTM in intact *Lymnaea* (Smyth et al., 2002), and we have also previously shown in intact *Lymnaea* that four training sessions separated by 1 h leads to LTM 18 h later (Lowe and Spencer, 2002).

Initially, data are shown from experiments during which the pneumostome openings and total breathing activity of the preparations were assessed in 'freely behaving' pre- and post-test observation sessions (as conducted previously in *Lymnaea* semi-intact preparations; McComb et al., 2005). Initially a 20 min observation session (pre-test) was given to all preparations. During this pre-test session, all preparations were allowed to breathe freely (no stimulus was applied) and the total breathing time for each preparation during this session was calculated. The preparations were then randomly assigned to either be operantly conditioned ($N$=9) or to serve as yoked controls ($N$=9). Both groups then underwent the four sessions (conditioned or yoked) and memory test. Following the memory test, both the conditioned and yoked control preparations were then given another 20 min observation session (post-test). During the post-test, the conditioned and yoked controls were again allowed to breathe freely, and again the total breathing time was calculated. The data revealed that only the conditioned group showed a significant reduction in total breathing time from the pre to the post-test sessions (paired $t$-test; $t$=3.09, $P$<0.05; Fig. 2). In addition, the total number of pneumostome openings of the conditioned preparations were significantly reduced from pre-test (12.1±2.5) to post-test (0.66±0.67; paired $t$-test; $t$=4.308, $P$<0.01). Meanwhile the number of pneumostome openings in yoked controls was not significantly changed from the pre-test to the post-test (pre: 9.0±2.2; post: 2.9±1.0; paired $t$-test, $P$>0.05).

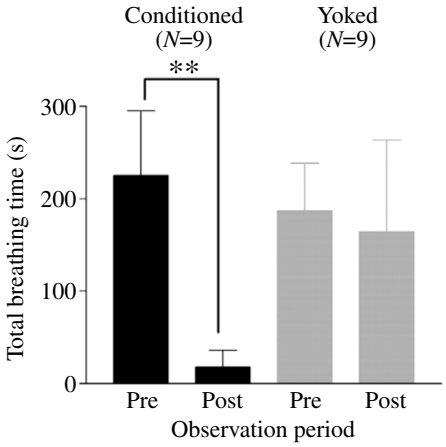


Fig. 2. Operantly conditioned semi-intact preparations demonstrated a reduction in total breathing following conditioning for learning and LTM. Conditioned and yoked control preparations were given a 20 min pre-observation period and a 20 min post observation period to assess total breathing time before and after their operant and yoked sessions, respectively. During the pre- and post-observation periods both conditioned and yoked preparations were allowed to breathe freely. The total breathing time of conditioned and yoked preparations during the pre-observation period did not significantly differ from each other (unpaired $t$-test=0.4451, $P$>0.05). Only the conditioned preparations demonstrated a significant reduction in total breathing time during the post observation period, which followed the LTM test (paired $t$-test=3.090, $P$=0.01). **$P$=0.01.

We also compared the number of attempted pneumostome openings made by the conditioned preparations ($N$=9) to the number of actual pneumostome openings of the yoked controls ($N$=9) during the training sessions and memory test. A two-way RM-ANOVA of these data showed a significant interaction effect (interaction $F_{(4,64)}$=2.635, $P$<0.05). *Post-hoc* analysis of these data confirmed that only the conditioned preparations demonstrated a significant reduction in attempted pneumostome openings between sessions 1 (15.89±2.389) and 4 (8.778±2.565; $t$=2.311, $P$<0.05) and between session 1 and the memory test (1.889±1.654; $t$=4.550, $P$<0.001), whereas the yoked controls showed no such changes ($P$>0.05). These experiments using the pre- and post-test observation sessions not only validated the yoked control procedure, but also indicated that recording the number of openings during the training sessions and memory test is an appropriate indicator of changes in behaviour, as shown previously (Lukowiak et al., 1996; McComb et al., 2005). Thus for all further experiments in this study, learning was operationally defined as a significant reduction in the number of attempted pneumostome openings of conditioned preparations between the first and last training sessions. LTM formation was operationally defined as a

significant reduction in attempted pneumostome openings between session 1 and the memory test, while the number of attempted openings between session 4 and the memory test 18 h later remain unchanged (Lukowiak et al., 1996; Lukowiak et al., 1998).

We next analysed the number of openings during training sessions of conditioned, yoked and naïve groups of preparations that did not undergo the pre- and post-test observation sessions. A two-way RM-ANOVA of the conditioned, yoked and naïve semi-intact data (Fig. 3A,B) revealed a significant interaction effect between the two independent variables (i.e. the treatment groups and training sessions/memory test; interaction $F_{(8,108)}$=2.145, $P$=0.0257). This result strongly indicated that the treatment paradigm had a significant effect on the respiratory behaviour of the conditioned preparations over the training sessions/memory test. *Post-hoc* analysis within each treatment group confirmed that following the four training sessions, only the conditioned preparations ($N$=10) demonstrated learning ($t$=3.481, $P$<0.01) and LTM in the memory test 18 h later ($t$=3.439, $P$<0.01; Fig. 3A). The yoked control ($N$=10) and naïve control ($N$=10) preparations did not show any significant change ($P$>0.05) in the number of pneumostome openings across sessions (Fig. 3B). Because the breathing behaviour of the yoked controls and naïve preparations was not interrupted by the aversive stimulus (as in the conditioned preparations), we were also able to monitor the total breathing time during each session and memory test. Neither the yoked nor naïve semi-intact preparations showed any significant change in their total breathing time across the sessions or memory test ($P$>0.05) and thus showed no evidence of learning or LTM (Fig. 3C). These data, together with the data from Fig. 2, confirm that contingent stimulation of the conditioned semi-intact preparation did indeed produce learning and LTM 18 h later, whereas the controls showed no significant change in respiratory behaviour over time (i.e. pneumostome openings or total breathing time).

### *Hypoxic controls*

It is possible that the conditioned semi-intact preparations experienced a greater amount of 'hypoxic stress' than that of the naïve and yoked control preparations. This is because the conditioned preparations were not given the opportunity to perform aerial respiration during the training sessions. However, despite preventing their aerial respiration, the conditioned preparations could still perform cutaneous air–gas exchange. To test whether cutaneous gas exchange was sufficient to maintain the viability of the preparation over the test period, we incorporated a hypoxic control group. We tested the ability of preparations placed in the hypoxic control group to perform aerial respiration before, and 18 h after five hypoxic stress sessions. It was shown that in the hypoxic control group ($N$=10) there was no significant reduction in the number of pneumostome openings (pre-test=10.0±2.7; post-test=5.7±1.9; paired $t$-test, $P$>0.05) or total breathing time (pre-test=230.1±73.8 s; post-test=316.1±118.3 s; paired $t$-test, $P$>0.05). These data confirm that the reduction in respiratory behaviour in conditioned preparations was not a result of hypoxic stress.

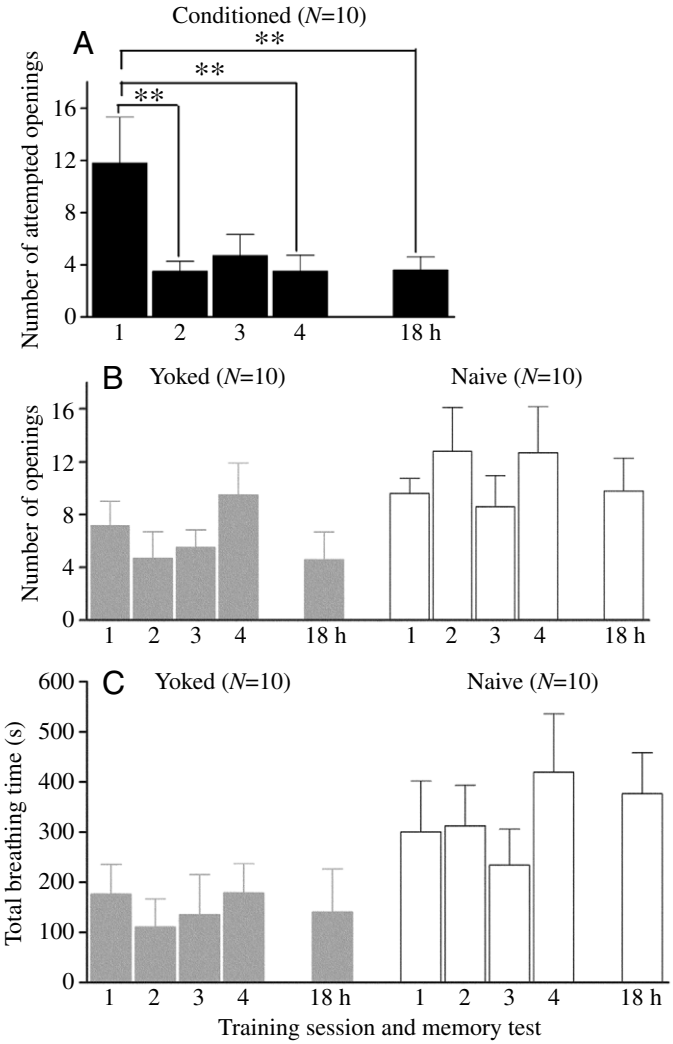


Fig. 3. Operantly conditioned semi-intact preparations demonstrated learning and long-term memory, but yoked and naïve preparations did not. The number of openings (and total breathing time for controls) were assessed during each session. (A) Conditioned preparations showed a significant reduction in attempted pneumostome openings when comparing session 1 to 4 (learning; $t$=3.583, $P$<0.01) and session 1 to the memory test (LTM; $t$=3.539, $P$<0.01). In addition, there was also a significant reduction in attempted pneumostome openings in the conditioned group between session 1 and 2 ($t$=3.280, $P$<0.01). **$P$<0.01. (B) A two-way RM-ANOVA demonstrated that the number of pneumostome openings in yoked (grey bars) and naïve preparations (white bars) remained unchanged across the training sessions and in the memory test 18 h later ($P$>0.05 for treatment, sessions and interaction effects). There was no significant difference in number of openings in Session 1 across all three groups ($P$>0.05). (C) Although the total breathing time of yoked preparations was lower than that of the naïve preparations ($F_{(1,18)}$=5.879, $P$<0.05) there was no significant decline in the total breathing time within each treatment group when comparing session 1 to 4 (yoked: $t$=0.0303, $P$>0.05; naïve: $t$=1.115, $P$>0.05) and session 1 to the memory test (yoked: $t$ =0.401, $P$>0.05; naïve: $t$=0.7139, $P$>0.05).

*Semi-intact preparations given only two training sessions demonstrated learning, but not LTM*

From the conditioned data shown in Fig. 3A, it was noted that in addition to a significant decrease in attempted pneumostome openings from session 1 to session 4 there was also a significant reduction in openings from session 1 to session 2 ($t$=3.481, $P$<0.01). This prompted us to determine whether two training sessions were sufficient to produce learning and LTM in the semi-intact preparation. To address this question, a separate group of conditioned and yoked preparations were given only two training sessions (separated by 1 h), followed by the memory test 18 h later. A two-way RM-ANOVA of these data revealed a significant interaction effect between the two independent variables (i.e. treatment groups and training sessions; interaction $F_{(2,54)}$=3.087, $P$<0.01). Only the conditioned group ($N$=10) showed a significant reduction in the number of pneumostome openings between sessions 1 and 2 ($t$=2.711, $P$<0.05) to indicate that learning had occurred. However, there was no evidence of LTM 18 h later ($t$=0.1196, $P$>0.05; Fig. 4). The yoked control group did not show any change in either pneumostome openings (Fig. 4) or total breathing time (one-way RM-ANOVA, $F_{(2,18)}$=2.975, $P$>0.05; data not shown) across the two sessions and memory test. From this, we concluded that two training sessions were sufficient for learning to occur in the conditioned group, but not for LTM 18 h later.

*Investigating the neural basis of learning and LTM formation*

The experiments described so far demonstrate that the aerial respiratory behaviour of *Lymnaea* can be operantly conditioned *in vitro* to exhibit both learning and LTM. Thus we have a unique system for manipulating the neural activity during learning and LTM formation. Previous research has suggested that reduced activity in RPeD1 may play a role in LTM formation (Spencer et al., 1999; Spencer et al., 2002). Until now, however, there has been no way to directly investigate the relationship between changes in RPeD1 impulse activity and LTM formation in *Lymnaea*. Therefore, to directly investigate the role of RPeD1 activity in learning and LTM formation, its impulse activity was manipulated in conditioned preparations. Since the delivery of the reinforcing stimulus has been shown to lead to an overall reduction in the spontaneous impulse activity of RPeD1 in semi-intact preparations previously conditioned for LTM (Spencer et al., 2002), we hypothesized that suppressing its activity may augment learning and/or the formation of LTM. Thus, using the two-session training paradigm described above, our next aim was to determine the effects of inhibiting the impulse activity of RPeD1 during the interval between the two training sessions. This manipulation was carried out at this time point for two reasons. Firstly, as RPeD1 is the cell responsible for initiating the CPG activity (Syed et al., 1990), it would not be feasible to suppress its activity during the training period without affecting its ability to initiate the behaviour. Secondly, the time interval between the training sessions is known to be important for memory consolidation in *Lymnaea* (Lukowiak et al., 2000; Sangha et al., 2003a), but no studies have previously investigated the effects of manipulating cellular activity during this period.

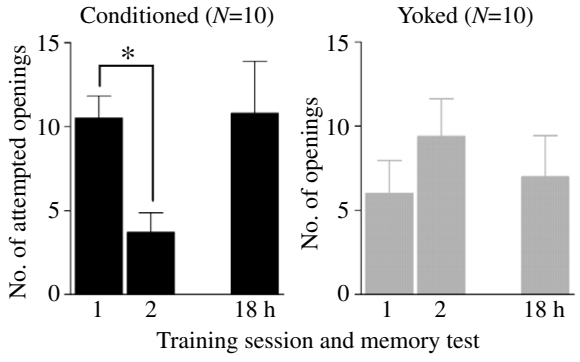


Fig. 4. Operantly conditioned semi-intact preparations given only two training sessions demonstrated learning, but not long-term memory. Conditioned preparations demonstrated a significant reduction in attempted pneumostome openings between session 1 and 2 ($t$=2.711, $P$<0.05) indicating learning, but not between session 1 and the 18 h memory test ($t$=0.1196, $P$>0.05). *$P$<0.05.

*Sham controls*

Prior to carrying out these manipulations, conditioned sham controls were first devised to determine whether merely impaling the soma of RPeD1 immediately following session 1 (to record impulse activity only), would adversely affect the respiratory behaviour of the animal. Specifically, we aimed to confirm that electrode penetration did not affect learning in the conditioned preparations. Naïve sham controls were also included, however, in order to show that the respiratory behaviour in freely behaving preparations was also not affected. The soma of RPeD1 was impaled with a microelectrode and impulse activity was recorded for 20 min immediately after session 1 (Fig. 5A,B), after the saline level had been raised. Hyperpolarizing current was not injected in these preparations. A two-way RM-ANOVA of these data indicated that a significant interaction effect occurred between the two factors (i.e. treatment group and training sessions/memory test; interaction $F_{(2,36)}$=6.8158, $P$=0.0013). As might be expected from the above experiments (Fig. 4), the operantly conditioned sham controls ($N$=10) showed a significant reduction in attempted pneumostome openings between sessions 1 and session 2 to indicate learning ($t$=2.818, $P$<0.05). However, they did not demonstrate LTM 18 h later (Fig. 5C). Naïve sham controls ($N$=10) showed no reduction in pneumostome openings (Fig. 5C) or total breathing time (data not shown) either across the two sessions or in the memory test. Furthermore, we found no significant difference in the resting membrane potential of RPeD1 in the conditioned sham controls (–58.9±1.1 mV) and the naïve sham controls (–59.3±0.6 mV; unpaired $t$-test, $t$=0.3196, $P$>0.05). These data showed that merely impaling RPeD1 with a microelectrode between the two training sessions did not affect learning or LTM formation in the conditioned sham preparations.

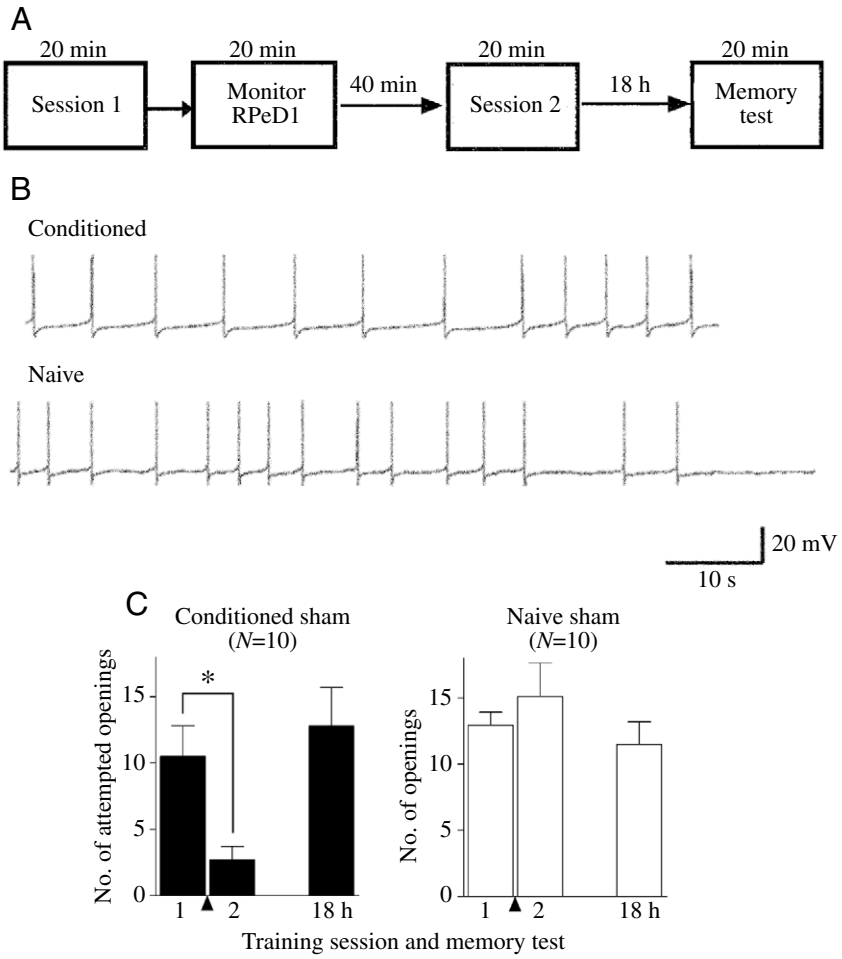


Fig. 5. Operantly conditioned sham controls demonstrated learning, but not LTM. Conditioned sham controls were conditioned preparations (two sessions only) in which RPeD1 was impaled by an electrode but was not hyperpolarized, in order to determine if impaling the soma of RPeD1 with sharp electrodes affected the preparation's behaviour. (A) The soma of RPeD1 was impaled for up to 20 min immediately following the completion of training session 1. (B) Sample recordings of RPeD1 impulse activity recorded in conditioned and naïve preparations after the completion of training session 1, after the saline level had been raised. (C) Impaling RPeD1 with sharp electrodes did not affect behaviour, since the conditioned sham preparations still showed learning between session 1 and 2 ($t$=2.818, $P$<0.05), but no LTM was formed ($t$=0.8309, $P$>0.05). Within C, arrowheads denote time of electrode insertion. *$P$<0.05.

### *Perturbation of RPeD1 impulse activity augmented LTM formation*

Animals were randomly assigned to either the conditioned, yoked, or naïve test groups prior to dissection. All test groups were given only two 20 min sessions, followed by an LTM test 18 h later (Fig. 6A). Immediately following the completion of session 1, RPeD1 impulse activity was prevented for 20 min in each test group (Fig. 6B). The second session commenced 40 min following manipulation of the neural activity.

A two-way RM-ANOVA of these data indicated that a significant interaction effect occurred between the two factors (i.e. treatment group and training sessions/memory test; interaction $F_{(4,66)}$=3.538, $P$<0.05). Conditioned preparations ($N$=12) given only two training sessions, coupled with inhibition of impulse activity in RPeD1, showed a significant reduction in their number of attempted pneumostome openings between sessions 1 and 2 ($t$=4.014, $P$<0.001; Fig. 6C) to indicate learning. In addition, these conditioned preparations also showed a significant reduction in the number of attempted openings between session 1 and the memory test ($t$=4.176, $P$<0.001; Fig. 6C). Thus, conditioned preparations not only demonstrated learning, but now also showed LTM 18 h later.

Naïve preparations, in which RPeD1 impulse activity was prevented, also showed a significant reduction in pneumostome openings ($t$=3.733, $P$<0.01; Fig. 6C) and in total breathing time ($t$=2.997, $P$<0.05; data not shown) from session 1 to session 2. However, this effect was transient and did not produce LTM, as no significant reduction in respiratory behaviour occurred between session 1 and the memory test ($t$=0.6616, $P$>0.05). Meanwhile, the hyperpolarizing current injection did not alter the number of pneumostome openings (Fig. 6C) or the total breathing time (data not shown) of the yoked control preparations ($N$=12). We also found no significant difference in the resting membrane potential of RPeD1 in the conditioned (–60.2±1.3 mV), yoked (–59.7±0.5 mV) and naïve (–57.7±1.1 mV) preparations that received the hyperpolarizing current injection (one-way ANOVA, $F_{(2,33)}$=1.638, $P$>0.05).

Taken together, these data indicate that inhibition of RPeD1 impulse activity between the two training sessions augmented the formation of LTM in conditioned preparations only.

### *Increasing RPeD1 impulse activity did not augment LTM formation*

Having shown that preventing RPeD1 impulse activity between training sessions augmented the formation of LTM in conditioned preparations, we then decided to investigate the behavioural outcome of enhancing its impulse activity. This experimental manipulation was performed only to determine

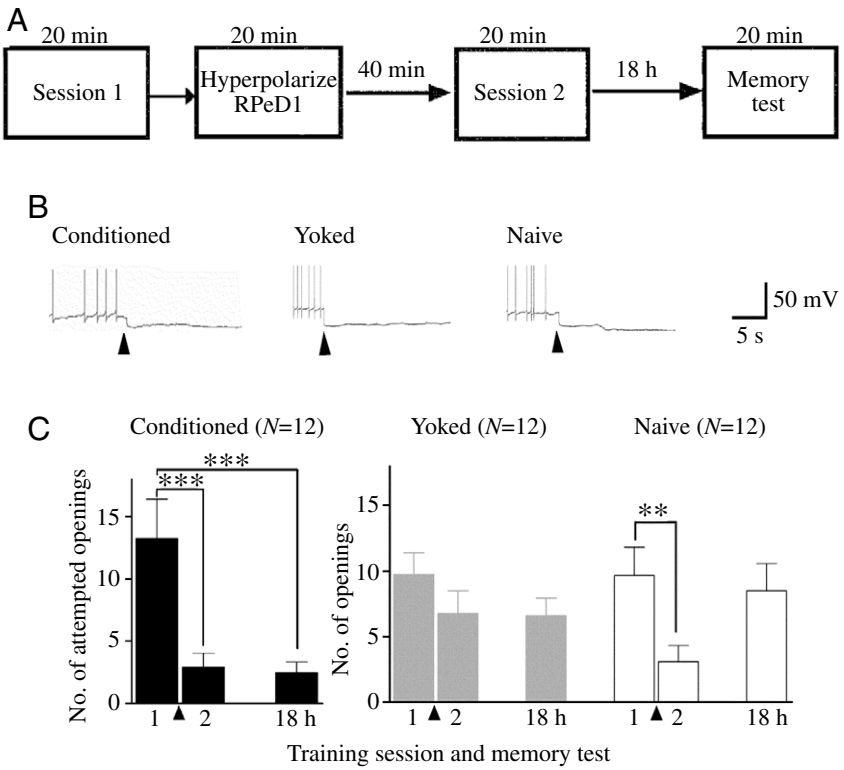


Fig. 6. Preventing RPeD1 impulse activity in operantly conditioned preparations augmented the formation of long-term memory. (A) Upon completion of session 1 in conditioned, yoked, and naïve control preparations, RPeD1 was penetrated with a microelectrode followed by the immediate injection of hyperpolarizing current to prevent impulse activity for up to 20 min. (B) Sample recordings in which RPeD1 impulse activity was prevented after the completion of training session 1 (arrowheads denote the time of hyperpolarizing current injection in B and C). (C) Conditioned preparations significantly reduced their number of attempted pneumostome openings between sessions 1 and 2 (learning; $t$=4.014, $P$<0.001) and session 1 and the memory test 18 h later (LTM; $t$=4.176, $P$<0.001). Naïve preparations also significantly reduced their pneumostome openings in session 2 ($t$=3.733, $P$<0.01), but not in the memory test ($t$=0.6616, $P$>0.05) and thus did not show LTM. **$P$<0.01; ***$P$<0.001.

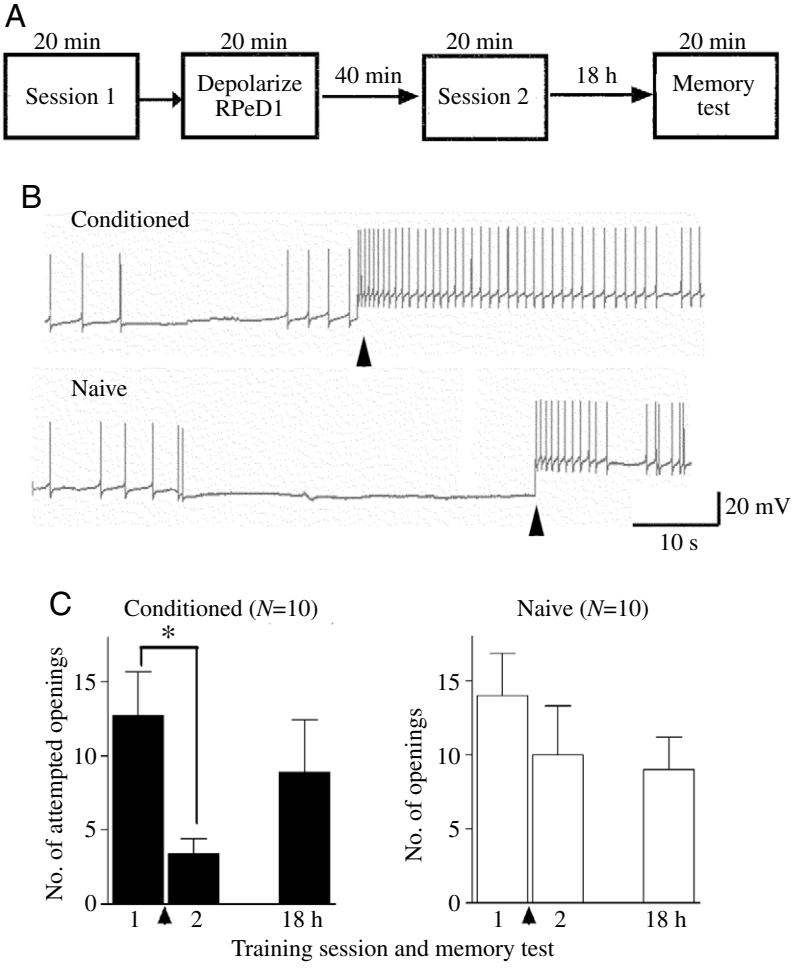


Fig. 7. Increasing RPeD1 impulse activity did not augment long-term memory formation in operantly conditioned preparations. (A) In conditioned and naïve preparations RPeD1 was depolarized (i.e. by injecting positive current) for up to 20 min immediately after the completion of session 1 (and after saline level had been raised). (B) Sample recordings in which RPeD1 impulse activity was increased in conditioned and naïve preparations after the completion of training session 1 (arrowheads denote the time of the depolarizing current injection in B and C). (C) Only the conditioned preparations significantly reduced their number of attempted pneumostome openings between sessions 1 and 2 (learning; $t$=2.477, $P$<0.05), but not between session 1 and the memory test 18 h later (LTM; $t$=1.012, $P$>0.05).

whether augmentation of LTM was specific to hyperpolarization or whether another manipulation (e.g. depolarization) could produce the same result. In these experiments, RPeD1 was depolarized to increase impulse activity for 20 min, immediately following the completion of the first training session (Fig. 7A). The amount of depolarizing current injected (0.4 nA) was based on the minimum amount of hyperpolarizing current needed to prevent RPeD1 impulse activity in the previous experiments. Depolarizing RPeD1 in conditioned preparations significantly increased the firing frequency (1.42±0.19 Hz; $N$=10) throughout the 20 min duration of the current injection compared to that of conditioned sham (0.59±0.04 Hz, $N$=10) and naïve sham controls (0.52±0.10 Hz, $N$=10), both of which did not receive any current injection (interaction $F_{(2,72)}$=2.208, $P$=0.0474). Therefore, depolarization of RPeD1 in conditioned preparations with 0.4 nA of positive current was sufficient to significantly increase the frequency of impulse activity for the 20 min duration of each trial.

*Post-hoc* analyses of the conditioned preparations ($N$=10) in which RPeD1 was depolarized (Fig. 7B), showed a significant reduction in their number of attempted pneumostome openings from session 1 to 2, indicating that learning had occurred ($t$=2.477, $P$<0.05). However, these conditioned preparations did not demonstrate LTM 18 h later ($t$=1.012, $P$>0.05; Fig. 7C). Naïve preparations ($N$=10) were also tested to ensure there was no change in the behaviour of freely behaving preparations when RPeD1 was depolarized (Fig. 7B). In naïve preparations there was no significant change in their number of pneumostome openings (Fig. 7C) or total breathing time ($P$>0.05; data not shown) across the two training sessions and memory test 18 h later. These data indicate that augmentation of LTM was specific to hyperpolarization of RPeD1 and could not be caused by a depolarizing manipulation.

In summary, conditioned semi-intact preparations that received four 20 min training sessions followed by a memory test, demonstrated both learning and LTM *in vitro*. Two 20 min training sessions alone were sufficient for learning, but not for LTM formation. However, when the impulse activity of RPeD1 was prevented in conditioned semi-intact preparations, two training sessions produced both learning and LTM. Increasing the activity levels of RPeD1 had no obvious effect.

## Discussion

Recent studies that have made significant advances in understanding the neural basis of operant conditioning employed *in vivo* manipulations of the molluscan CNS (Scheibenstock et al., 2002; Sangha et al., 2003b; Sangha et al., 2003c). Meanwhile, elucidating changes in neural activity (Spencer et al., 1999; Spencer et al., 2002) or biophysical properties (Brembs et al., 2002) of identified neurons has only been characterized following dissection of intact animals previously operantly conditioned for LTM. Here we report, for the first time, the operant conditioning of a long-lasting molluscan semi-intact preparation to demonstrate learning and LTM formation *in vitro*.

An obvious advantage of our long-lasting semi-intact preparation is that it allowed direct manipulation of an identified CPG neuron, RPeD1, during the operant conditioning procedure for LTM. This alleviated some of the issues that hinder the ability to relate specific neural changes with specific behavioural changes seen in the intact animal. An added advantage of the semi-intact preparation over intact animals is the direct accessibility of the pneumostome area for delivery of the aversive stimulus to both the conditioned and yoked controls. For example, in whole animal experiments it is more difficult to directly stimulate the pneumostome of yoked controls, as they are not performing aerial respiration and the pneumostome area is often shielded by the shell (Lukowiak et al., 1996). In our semi-intact preparations, the pneumostome is completely exposed and the yoked controls always received the aversive stimulus directly to the pneumostome area. Though stimulated in the same location on the pneumostome, we cannot claim that yoked and conditioned preparations received stimuli of identical magnitude (due to the mechanical nature of the stimulus). However, it has previously been shown (Lukowiak et al., 1996) that increasing the strength of the stimulus to yoked preparations did not affect behaviour. Thus, though slight differences in stimulation strengths may have occurred, we feel it is unlikely that stimulus strength affected the outcome of these experiments.

An important consideration in the use of our long-lasting semi-intact preparation, was whether reduced behavioural activity was actually a result of the conditioning paradigm or general run-down of the preparation. *Lymnaea* performs air–gas exchange either cutaneously or aerially *via* its pneumostome (Jones, 1961) and exposure to hypoxic aquatic conditions is well known to increase *Lymnaea*'s aerial respiratory behaviour (Syed et al., 1991; Lukowiak et al., 1996; Taylor et al., 2003). It has been well documented that neither chronic exposure to hypoxia, nor preventing the animal from accessing the air–water interface for an extended period of time (i.e. by a physical barrier), adversely affects the ability of intact snails to perform aerial respiration (Lukowiak et al., 1996; Sangha et al., 2003a). Indeed, we confirmed that pneumostome openings and total breathing time of naïve hypoxic control preparations did not significantly change 18 h after exposure to hypoxia. These data further confirmed that conditioned preparations showed reduced pneumostome openings due to conditioning for LTM and not due to a diminished state of health from hypoxia.

With our robust preparation we were able to prevent RPeD1 activity between the training sessions and determine the behavioural outcome on memory 18 h later. Preventing the impulse activity of an identified neuron has previously been shown to abolish modulation of a reflex circuit in *Aplysia* (Wright and Carew, 1995). Furthermore, hyperpolarization of a single cell in *Lymnaea* has previously reduced conditioning-induced responses (Jones et al., 2003). In contrast, we report here enhanced memory formation by hyperpolarizaion of an identified neuron, RPeD1, between training sessions. The

importance of this time interval between training sessions for LTM consolidation in *Lymnaea* has been well established (Lukowiak et al., 2000), but no previous studies have investigated either RPeD1 or CPG activity during this consolidation period. Here we show, for the first time, that preventing RPeD1 activity between training sessions can directly augment LTM formation. It must be noted, however, that preventing RPeD1 impulse activity in naïve preparations also produced a transient reduction in respiratory behaviour between sessions 1 and 2, whereas naïve sham controls (no hyperpolarization) showed no such change. This finding suggests that merely hyperpolarizing RPeD1 (in the absence of conditioning) had short-term effects to reduce behaviour, which is not surprising when we consider that RPeD1 activity initiates the respiratory CPG rhythm (Syed et al., 1990). Others have also shown a change in behaviour of naïve preparations as a result of manipulating the activity of a single identified neuron (Jones et al., 2003). It is currently unclear as to why RPeD1 hyperpolarization did not also produce a transient reduction in behaviour in the yoked controls. We can only speculate that the non-contingent presentation of the stimulus somehow interfered with the transient effects on behaviour arising from RPeD1 hyperpolarization.

One important consideration of these studies is that preventing somal activity in RPeD1 may not necessarily affect the synapses located distal from the cell body. Thus we cannot completely rule out that local depolarizations at distal synaptic sites may still be occurring and potentially playing a role in LTM formation. Furthermore, the same point applies to the control depolarizing stimulus applied to the soma, which did not augment LTM formation. It is possible that spikes induced in the soma did not invade the distal synaptic sites. However, we clearly showed that augmentation of LTM was induced by hyperpolarization to the somatic compartment and that a depolarizing stimulus to the same region of the cell did not produce the same effect. It is possible that our hyperpolarizing stimulus affected only synapses terminating immediately proximal to the soma of RPeD1 and/or possibly reduced activity-dependent gene expression in RPeD1. LTM formation in *Lymnaea* has previously been shown to require gene activity in RPeD1's soma (Scheibenstock et al., 2002), though it is not yet known whether this activity reflects up- or downregulation of genes.

At present, it is not known exactly how the 20 min hyperpolarization of RPeD1 in the interval between training sessions augmented LTM formation in our conditioned preparations. Application of the aversive reinforcing stimulus produces inhibition of RPeD1 firing; thus it is plausible that our hyperpolarizing stimulus to RPeD1 in some way 'mimics' the aversive stimulus and extends the duration of the training period at the neuronal level. If this is the case, it is possible that this 'artificial reinforcement' may result in enhanced memory formation. Though the cellular and molecular mechanisms mediating this effect are currently unknown, we propose that changes in the activity of RPeD1 ultimately affect the respiratory network properties, possibly in a subtle and widely distributed manner.

The concept that changes in neuronal excitability play an important role in encoding information is not a new one, and has its origins in invertebrate systems (reviewed by Daoudal and Debanne, 2003). However, it is gaining new attention and a recent review (Giese et al., 2001) suggests that modulation of neuronal excitability is an essential mechanism for learning and memory. Though most examples in the literature cite increased neuronal excitability in mnemonic processes, there is also precedence for reduced firing, inhibition and hyperpolarization. For example, it has recently been shown that in addition to increasing the excitability of a single neuron (S-cell) involved in sensitization, 5HT also reduces the excitability of the same neuron, presumably through different receptors (Burrell et al., 2001). They proposed that 5HT-induced inhibition of the S-cell may be involved in habituation, which also decreases S-cell excitability. Evidence from vertebrate preparations indicates that sustained changes in firing levels can occur as a result of both depolarizing and hyperpolarizing stimuli. Specifically, in entorhinal cortical neurons, repetitive application of hyperpolarizing current pulses, as well as synaptic inhibition, led to graded and stable decreases in neuronal firing rates (Egorov et al., 2002). Interestingly, brief hyperpolarizations were ineffective in producing such changes. These authors propose that graded changes in cellular activity may form an elementary mnemonic process, and though the sustained increases in firing may be more important in their system, the experiments clearly demonstrate that hyperpolarization and/or inhibitory inputs produce similar graded reductions in activity. Such reductions in activity may prove equally important in other systems. For example, in our *Lymnaea* preparation, it is feasible that continual application of the aversive stimulus during conditioning may eventually lead to a sustained reduction in RPeD1 firing (Spencer et al., 1999; Spencer et al., 2002), which may ultimately affect network properties to produce long-term changes in behaviour. Future studies will examine in greater depth exactly how the firing properties of RPeD1 affect LTM. In the meantime, it is clear from this and previous work (Spencer et al., 1999; Spencer et al., 2002), that the level of RPeD1 activity is important in the operant conditioning of the aerial respiratory behaviour in *Lymnaea*. Interestingly, in juvenile *Lymnaea*, RPeD1 spontaneous activity is higher than in adults, and these juveniles are not capable of forming LTM (McComb et al., 2003).

Finally, as RPeD1 is a dopaminergic neuron, the fact that its activity is reduced following aversive training is consistent with other studies supporting a role for dopamine in reward learning. For example, dopamine plays an important role at the level of a single neuron (B51) during an operant reward paradigm in the invertebrate *Aplysia* (Brembs et al., 2002). Furthermore, it has recently been shown that dopamine neurons in the rat CNS consistently show reduced firing and reduced bursting activity following aversive stimuli (Ungless et al., 2004). These studies, together with many others, strongly support the notion that the role of dopamine in reward/punishment paradigms is strongly conserved across species.

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