The Journal of Experimental Biology 207, 2621-2630
Published by The Company of Biologists 2004
doi:10.1242/jeb.01073

Learning and memory in *Lymnaea* are negatively altered by acute low-level concentrations of hydrogen sulphide

David Rosenegger, Sheldon Roth and Ken Lukowiak*

Calgary Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

*Author for correspondence (e-mail: lukowiak@ucalgary.ca)

Accepted 4 May 2004

Summary

Hydrogen sulphide (H_2S) is a common industrial pollutant as well as an endogenous neural transmitter/neural modulator. Experiments were performed on the pond snail *Lymnaea stagnalis* to determine the acute effects of low-level exposure to H_2S (50–100 μmol l^{-1}) on aerial respiratory behaviour, associative learning, and its subsequent consolidation into long-term memory (LTM). A 3-neuron network whose sufficiency and necessity have been demonstrated drives aerial respiratory behaviour in *Lymnaea*. In the presence of 100 μmol l^{-1} H_2S the number of bouts of aerial respiration and the total breathing time were significantly increased compared to the control hypoxic situation, but were equivalent to those observed in snails that had been subjected to a 'more intense hypoxic challenge'. In addition, at a concentration of 100 μmol l^{-1} H_2S neither associative learning nor long-term memory (LTM) were observed. However, snails subjected to a 'more intense hypoxic challenge' still had the capacity to learn and form LTM. These snails, in fact, showed statistically the best learning and memory performance of any group. While learning and memory were observed at 50 and 75 μmol l^{-1} H_2S, respectively, they were statistically poorer than the learning and memory exhibited by snails in the standard hypoxia condition. Hence the ability to learn and form memory was compromised by H_2S. Thus an invertebrate model system with a well-defined neural network can be used to study of the effects of H_2S on the processes of learning and memory.

Key words: hydrogen sulfide, *Lymnaea stagnalis*, operant conditioning, aerial respiratory behaviour, learning, memory.

Introduction

Hydrogen sulphide (H_2S) is regarded both as an environmental and an industrial pollutant (Roth, 1993); and while it is capable of affecting many different organ systems, the brain is considered to be one of its primary, critical targets (Reiffenstein et al., 1992; Roth, 1999, in press). Although there are considerable data addressing the effects of acute, high-dose exposure to H_2S, there are substantially less data on the effects of chronic low-level exposure to H_2S on cognitive brain functions, such as learning and memory (Roth, 1999). Acute exposure to H_2S in human subjects results in a wide variety of effects, including dizziness, lack of coordination, headache, loss of concentration, difficulty in remembering and increased respiratory activity (Kilburn, 1997; Hessel and Melenka, 1999; Milby and Baselt, 1999). These and other studies point toward H_2S having adverse effects on important homeostatic behaviours, including the ability to learn and form memory. However, it is unclear how these deleterious effects are mediated at the neuronal level. Notwithstanding the number of clinical or anecdotal reports indicative of an unfavorable effect (i.e. cognitive dysfunction) of chronic, low-level exposure to H_2S (Roth, 1999), a systematic study of the mechanisms by which H_2S produces its deleterious effect on learning and memory remains to be undertaken.

Our understanding of the effects of H_2S on living organisms is quite limited. H_2S is itself an endogenous neurotransmitter/neural modulator across all animal phyla (Abe and Kimura, 1996; Julian et al., 2002; Eto and Kimura, 2003; Eto et al., 2002), and its actions have yet to be clearly delineated. A simpler model system is needed to directly determine where and how H_2S alters learning and memory. For example, although H_2S exposure may result in memory loss in rodents, at physiological concentrations H_2S facilitates long-term potentiation (LTP) in neuronal structures thought to be necessary for memory formation in an activity- and dose-dependent manner (Abe and Kimura, 1996; Kimura, 2000). We therefore have made use of a model system that was initially developed to elucidate the causal neuronal mechanisms of learning and memory (Lukowiak et al., 2003b) to examine the effects of low-level exposure to H_2S on a relatively simple, adaptable behaviour.

Our model system, the pond snail *Lymnaea stagnalis*, is a bimodal breather that satisfies its oxygen needs either cutaneously, *via* diffusion across the skin, or aerially, by opening the pneumostome (the respiratory orifice) at the water surface. It is therefore possible to modulate one of its respiratory behaviours while leaving the other unaffected. We

make use of a non-declarative, operant (i.e. instrumental) conditioning paradigm to decrease the occurrence of aerial respiratory behaviour (Lukowiak et al., 1996, 1998, 2000, 2003a). These snails can still breathe cutaneously and thus our procedure is not harmful to the animals.

Naïve snails when placed in hypoxic pondwater preferentially perform aerial respiration (Lukowiak et al., 1996). Snails placed in hypoxic pondwater are thus 'motivated' to perform aerial respiration and we operantly (instrumentally) condition snails by applying a tactile stimulus to the pneumostome area as they begin to open their pneumostome. Snails associatively learn not to perform aerial respiration and are capable of committing this learning into long-lasting *non-declarative* memory (Lukowiak et al., 2003a). By varying the length and number of the training sessions given, different forms of memory can be developed (Lukowiak et al., 2000). Intermediate term memory (ITM) lasts for only a few hours and requires *only* the translation of pre-existing mRNA, whereas long-term memory (LTM) lasts for at least 24 h and requires *both* the transcription of new mRNA along with its translation (Sangha et al., 2003a).

A major advantage of our model system is that the underlying neural circuitry controlling the behavior has been well characterized. A three-neuron central pattern generator (CPG) has been found to be both necessary and sufficient for controlling aerial respiration (Syed et al., 1990, 1992). Since non-declarative memories are stored within the neuronal circuit mediating the behaviour (Milner et al., 1998), and since the circuit that drives aerial respiratory behaviour is known as well as or better than any other neuronal circuit, we possess a clearcut advantage in determining the causal mechanisms of learning and its consolidation into memory. Neural correlates of learning and LTM have been shown in one of the CPG neurons, RPeD1 (Spencer et al., 1999, 2002), which has been directly demonstrated to be a necessary site for LTM formation, reconsolidation and extinction (Scheibenstock et al., 2002; Sangha et al., 2003b,c). The experimental advantages of our model system may allow us to directly determine how H_2S affects important homeostatic behaviours such as respiration and, importantly, how learning and its ability to be consolidated into memory are affected at the level of a single neuron.

Materials and methods

Snails

We used adult pond snails *Lymnaea stagnalis* L. (i.e. >20 mm in shell length), bred in facilities at the University of Calgary. Experiments were performed at room temperature (22–23°C), and the animals were maintained at this same temperature. Snails had continuous access to food (lettuce) in their eumoxic (i.e. normal levels of oxygen, ~ 6 ml O_2 l^{-1}) home aquaria. Adult snails show remarkable consistency in their ability to learn and remember when using the training procedures employed here (Lukowiak et al., 2003a).

All experiments were performed in a 1 liter beaker filled with 500 ml of hypoxic water. Experiments were performed under hypoxic conditions in order to increase the occurrence of the aerial respiration behaviour (i.e. opening and closing of the pneumostome, the respiratory orifice). Under eumoxic conditions there is no real need for the snails to surface and open their pneumostomes to breathe, so they cannot be easily conditioned. The water was made hypoxic (<0.1 ml O_2 l^{-1}) by bubbling nitrogen through it for at least 20 min prior to the beginning of the experiment.

The H_2S environment

The H_2S environment was created by dissolving a stock liquid Na_2S solution (iodometrically titrated to 75 mmol l^{-1}), into 500 ml of water, until the desired concentration was reached. The Na_2S ionizes in the water to form one third H_2S and two thirds HS^- (it is unknown which of these is the physiologically active form). The addition of the Na_2S solution did not alter the pH of the water. A fresh solution of the stock Na_2S and water was created for each session just prior to the commencement of acclimatization (see below) to ensure consistent concentrations. In these experiments conditions are said to be 'standard' (i.e. just hypoxic) when there is no H_2S present in the solution. During experiments involving H_2S, N_2 was only bubbled prior to the addition of H_2S to prevent its dissipation. Oxygen levels were tested to ensure that there was no significant difference between sessions that had continual nitrogen bubbling and those that only had the pre-bubbling.

Water oxygen levels

The oxygen content of the water under the various paradigms was determined using a Polarographic amplifier (A-M Systems model 1900, Sequin, WA, USA) and electrode. Readings (in nA) were taken while the water was saturated with either nitrogen or pure oxygen to act as controls, closely approximating 0% and 100% dissolved oxygen, respectively (the beakers were covered with Parafilm during these readings to prevent air from mixing with the water). Readings were then taken under experimental conditions including: (1) after bubbling N_2 for 30 min while still bubbling N_2, (2) after removing the air stone after 30 min of N_2 bubbling (i.e. no N_2 bubbling at time of measurement), (3) after adding the Na_2S (100 μmol l^{-1} final concentration) to the water that had been bubbled with N_2 for 30 min, (4) 45 min after Na_2S had been added to the water, and (5) water that is at equilibrium with the atmosphere. A linear relationship was created with the two standards, and was used to determine the approximate % oxygen saturation in the water.

Breathing observations

Snails were placed in a 1 liter beaker filled with 500 ml of hypoxic water for an initial acclimatization period of 10 min; they were then gently pushed just below the surface at the beginning of the observation period. The aerial respiratory behavior was monitored continuously for 45 min. After this initial observation period the snails were returned to eumoxic water for 1 h. A second 45 min observation period was then

performed in hypoxic water using 100 μmol l^{-1} of H_2S. A final 45 min breathing observation was performed 1 h later in standard hypoxic water. The time at which each snail opened and closed its pneumostome was recorded. From these recordings the number of pneumostome openings, total breathing time, and average breathing time per opening were calculated for each snail. Breathing observations that were carried out with H_2S were done at a concentration of 100 μmol l^{-1}, and a plastic cover was used to limit any gas dissipation.

A second set of breathing observations following a more 'intense hypoxic challenge' were also performed. In these observations the snails' breathing behaviour was first observed for 45 min in eumoxia. Following a 1 h interval in eumoxia they were placed under a barrier in hypoxic pondwater for a period of 45 min. They could not perform aerial respiratory behaviours for this period of time prior to the start of the second observation period. In these experiments there was no third observation period as previously we have shown that the breathing parameters return to control levels following this procedure (McComb et al., 2002). The 45 min submersion in hypoxic water significantly increases aerial breathing behaviour, presumably due to the accumulation of an oxygen deficit.

Operant conditioning

For training, snails are placed in 500 ml of hypoxic water. The snails are allowed a 10 min acclimatization period prior to the training session. The snails are then pushed below the surface of the water just before the training begins. Training involves the application of a tactile stimulus to the pneumostome of the snail when it attempts to perform aerial respiration. The time of each attempted opening is recorded. The training periods last for 30 or 45 min, with 1 h between sessions. Two training sessions are performed with a memory test 24 h later. The memory test (session 3) follows the same procedure as the training sessions. The operant conditioning training procedure used produces a long-term memory (LTM) that persists for at least 24 h (Lukowiak et al., 2003a,b).

Snails were subjected to H_2S at one of three times: (1) for 1 h prior to the first training session only; (2) during all training and memory test sessions; (3) for 1 h after each training session.

Another cohort of snails was also subjected to the 45 min hypoxia submersion procedure prior to both the operant conditioning training sessions and the test for savings 24 h later. These experiments were performed to determine if it was still possible for the snails to learn even with their increased motivation for aerial respiration.

Operational definitions of learning and memory

We have operationally defined memory as previously described (Lukowiak et al., 1996, 2003b; Sangha et al., 2003b,c). Learning was present if the number of attempted pneumostome openings in the last training session was significantly less than the number of attempted openings in the first training session. In order to be defined as memory, two criteria had to be met: (1) the number of pneumostome openings in the test for savings was significantly lower than that of the first training session, and (2) the number of pneumostome openings in the test for savings was not significantly higher than that of the last training session.

Yoked controls

Yoked controls were performed under standard hypoxic conditions, and hypoxic conditions with H_2S at a concentration of 75 μmol l^{-1}. These experiments were performed as previously described (Lukowiak et al., 1996). In sessions 1 and 2 for the yoked control procedure the animal received a tactile stimulus to the pneumostome area every time the snail to which it was 'yoked' attempted to open its pneumostome (i.e. in these sessions snails did not receive a reinforcing stimulus contingent on when they open their pneumostome). However, in the third session (i.e. memory test, MT), these yoked control snails now received a tactile stimulus each time they attempted to open their pneumostome (i.e. they received contingent stimulation). We compared the number of attempted openings in MT of the yoked control snails with the number of attempted openings in MT of the operantly conditioned snails. If the observed change in behaviour is due to an associative process (i.e. due to contingent presentation of the tactile stimulus to the pneumostome as it attempted to open), the number of attempted openings in the yoked control cohort should be significantly greater than the number of attempted openings in the operantly conditioned group.

Assignment of marks

Snails were given grades on an individual basis to show how well (or how poorly) they learned. The following grading scheme was used to assess learning: a snail that showed a 50% or greater reduction in attempted pneumostome openings from the first session to the second session was given an A, B was a 35–49.99% reduction, C was a 20–34.99% reduction, and F was assigned when a reduction of less than 20% was observed (see Lukowiak et al., 2003b).

Statistics

To determine whether the number of attempted pneumostome openings was significantly altered as a result of operant conditioning, repeated-measures one-way analysis of variance (ANOVA) was performed. If the ANOVA was significant ($P<0.05$), a *post hoc* Fisher's LSD *t*-test was performed to show which sessions were significantly different. The same test was performed in determining the significance of the yoked controls. A χ^2-test statistic was used to determine if the assigned marks were different for cohorts of snails exposed to H_2S or the 'more intense hypoxic challenge'. Correlated (paired) *t*-tests were performed to determine whether or not breathing behaviour was significantly altered by H_2S. A session difference was considered statistically significant if $P<0.05$. A *t*-test for separate groups was used to determine if the increase in respiration caused by H_2S was

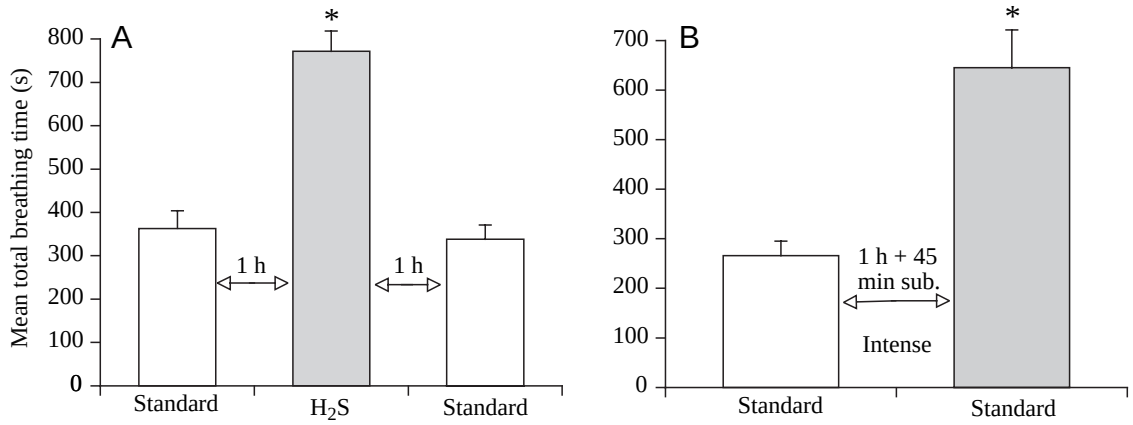

Fig. 1. Breathing observation experiments. (A) Three 45 min observations were performed, first in standard hypoxia, then in hypoxic H_2S (100 μmol l^{-1}), and lastly in standard hypoxic conditions again with 22 naïve snails. In between observation periods the snails were returned to eumoxic pondwater for 1 h. A significant increase in the second session *vs* the first session was seen (ANOVA $F_{(21,1)}$=98.5381, P<0.05), and there was no significant difference between sessions 1 and 3 (P>0.05). Session 2 was also significantly different when compared to session 3 (ANOVA $F_{(21,1)}$=65.8242, P<0.05). (B) A 45 min observation in standard hypoxia followed by 1 h in eumoxia, and then 45 min submerged in hypoxia (i.e. the 'more intense hypoxic challenge') and another 45 min observation period in standard hypoxia. The 'more intense hypoxic challenge' significantly increases the total breathing time (ANOVA $F_{(28,1)}$=31.6277, P<0.01). A separate group t-test between the breathing observation for the H_2S group (N=22) and the 'more intense hypoxic challenge' group (N=29) reveals that there is no statistical difference between the two (t=1.4211, P=0.1714 where d.f.=45.9649).

equivalent to the increase caused by the 'more intense hypoxic challenge' procedure.

Results

We first determined if aerial respiratory behaviour was significantly influenced by H_2S. Thus, aerial respiratory behaviour was measured (number of pneumostome openings, total breathing time and mean breathing time) in: (1) standard hypoxia; (2) hypoxic-H_2S condition (100 μmol l^{-1}); and (3) standard hypoxia again. Breathing behaviour was also similarly measured in another cohort of snails subjected to the 45 min submersion in hypoxia (i.e. the 'more intense hypoxic challenge').

Snails showed a statistically significant increase in the number of pneumostome openings, total breathing time and the average breathing time per pneumostome opening in the H_2S condition compared to standard hypoxia (Fig. 1A, Table 1). These observations also exemplify that aerial respiratory behaviour was not permanently altered by exposure to H_2S. That is, aerial respiratory behaviour recovered to pre-exposure levels following exposure to H_2S [NSD (no significant difference) session 1 *vs* 3, P>0.05; session 2 significantly different from sessions 1 and 3, P<0.01, for both comparisons].

It was possible that the increase in aerial respiratory behaviour in the hypoxic-H_2S condition was due not to an effect of H_2S but rather due to a decrease in O_2 content of the pondwater. Thus, oxygen levels of the solutions were measured to ensure that the increase in breathing behaviour observed under H_2S conditions, were not due to a chemical reaction that reduced the oxygen concentration in the water (Table 2). The recordings show that the addition of Na_2S to the water does not alter the oxygen levels of the water. The % O_2 saturation of the water does also not significantly increase 45 min after the cessation of N_2 bubbling.

We conclude from these experiments that acute exposure to H_2S significantly increases aerial respiratory behaviour of snails under hypoxic conditions, and that the breathing behaviour returns to normal afterward. Breathing behaviour was also significantly increased in the snails that were subjected to the 'more intense-hypoxic challenge' (mean total breathing time increased to 645.5 s from 266.2 s) compared to standard hypoxia (P>0.01). However breathing behaviours were not statistically different between this group and the snails subjected to hypoxia + H_2S (P=0.1714). Further, the elevated breathing was not due to H_2S decreasing the amount of oxygen in the water.

Having demonstrated that H_2S significantly but reversibly

Table 1. *Mean values of breathing observations*

Observation session (N=22)	Mean number of openings	Mean total breathing time (s)	Mean breathing time per opening (s)
1. Hypoxic	9.0	363.1	41.9
2. Hypoxic+H_2S (100 μmol l^{-1})	13.6	772.1	58.0
3. Hypoxic	8.1	338.1	41.6

Table 2. *Water oxygen levels*

Experimental condition	Sensor reading (nA)	Approx. % O_2 saturation of water
Extended N_2 bubbling (sealed)	0.08	0
Pure O_2 bubbling (sealed)	–8.13	100
After 30 min N_2 bubbling (still lightly bubbling)	–0.06	4
After 30 min N_2 bubbling (not bubbling anymore)	–0.09	4
After 30 min N_2 bubbling and Na_2S added	–0.12	4
45 min after addition of Na_2S	–0.25	5
Equilibrium with atmosphere	–1.64	21

increased aerial respiratory behaviour we wished to determine if H_2S affected the capability of snails to learn and/or form memory. Snails trained in the standard hypoxic condition exhibited learning and memory (Fig. 2A). We then determined the effect of operant conditioning training (two 45 min training sessions separated by a 1 h interval) on snails in 100 μmol l^{-1} H_2S-hypoxic pondwater (Fig. 2C). Snails trained under such conditions neither learned nor formed memory. That is, when we trained snails in 100 μmol l^{-1} H_2S-hypoxic pondwater there was no significant difference in the number of attempted pneumostome openings between session 1 and session 2. On the following day we tested the unlikely possibility that these snails formed memory. They did not. That is, the number of attempted pneumostome openings in session 3 was not significantly different from session 1. Notice in these experiments that snails still had the capacity to perform aerial respiration; however, they did not have the capability of making an association (i.e. learn) between opening the pneumostome and receiving a noxious stimulus.

It might be argued that the inability to learn and form LTM in the 100 μmol l^{-1} H_2S-hypoxic pondwater was due to the increased need to perform aerial respiration. To control for this

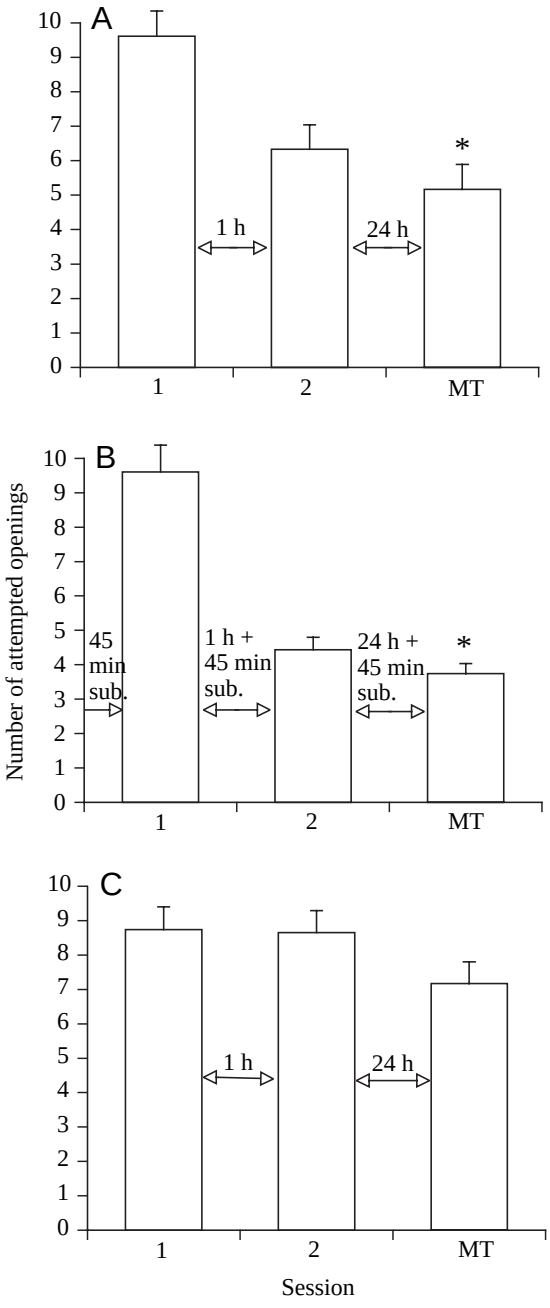

Fig. 2. Long-term memory (LTM), operant conditioning in 'standard' and H_2S environments. (A) 18 naïve snails received operant conditioning training under standard conditions (i.e. two 45 min sessions separated by 1 h) with a memory test (MT) performed 24 h later. This cohort exhibited both learning and memory. Learning was shown as the second training session was significantly lower than the first, and memory was shown as the memory test (MT) was significantly lower than the first session but not significantly greater than the second training session (ANOVA $F_{(17,2)}$=20.9304, P<0.01; sessions 2 and 3 are significantly different from session 1, P<0.01) (*signifies that a session is significantly different from session 1 but not from session 2). (B) A cohort of 23 snails that received the 'more intense hypoxic challenge' also demonstrated learning and memory. That is, since the number of openings in session 2 was significantly less than in session 1 (ANOVA $F_{(22,2)}$=40.6394, P<0.01) learning was demonstrated. Additionally, since the memory test session (MT) was significantly different from session 1 but not different from session 2 memory was shown (ANOVA $F_{(22,2)}$=66.8919, P<0.01 and $F_{(22,2)}$=2.5811, P=0.1224, respectively). (C) A separate cohort (N=23) of snails underwent the training and testing protocol in hypoxia + H_2S (100 μmol l^{-1}). These snails showed neither learning nor memory. That is, the data in session 2 were not significantly different than that in session 1 (i.e. no learning) and the memory test session (MT) was not significantly different from session 1 (i.e. criteria for memory not met; ANOVA $F_{(22,2)}$=2.3095, P=0.1112; no significant difference between sessions).

possibility we trained snails that were subjected to the 'more intense-hypoxic challenge'. These snails performed aerial respiration at the same level as the snails in 100 μmol l^{-1} H_2S-hypoxic pondwater did (Table 1). However, snails subjected to the 'more intense-hypoxic challenge' still have the capacity to learn and to form LTM (Fig. 2B). That is, the number of attempted pneumostome openings in session 2 was significantly less than in session 1 (i.e. learning demonstrated). Moreover, as the number of attempted openings in the savings test session was not statistically greater than the number in session 2, but was significantly less than the number in session 1, memory was shown. Thus the learning impairment caused by H_2S is not simply due to the fact that there is an increased drive for respiration.

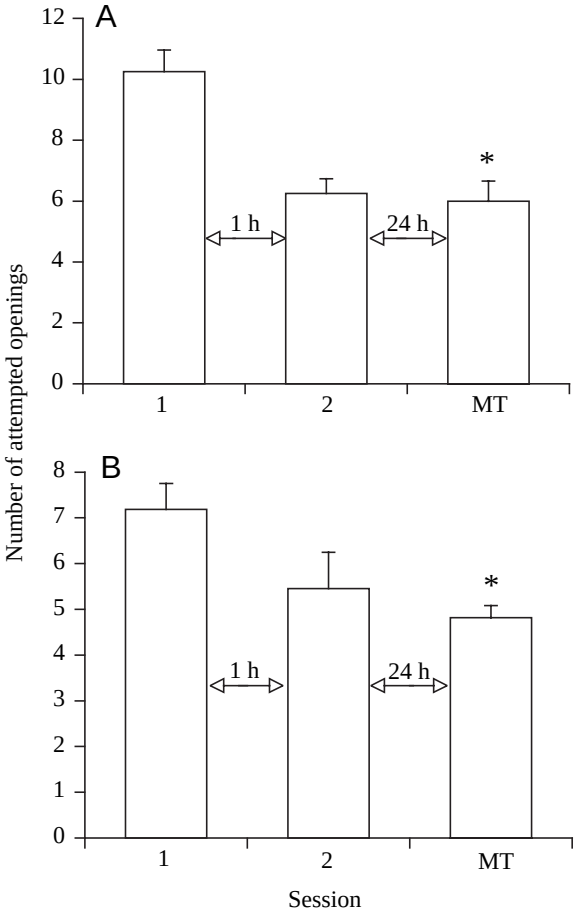

Fig. 3. Learning and memory of snails trained in 50 and 75 μmol l^{-1} of H_2S. (A) A cohort of naïve snails (N=24) were trained using the same LTM training procedure as in Fig. 2 while being exposed to H_2S at a concentration of 50 μmol l^{-1}. This cohort of snails showed both learning and memory (ANOVA $F_{(23,2)}$=23.9962 P<0.01; sessions 2 and 3 significantly different from session 1, P<0.01). (B) Another cohort of naïve snails (N=11) was subjected to the same LTM training procedure as in A except that these snails were exposed to an H_2S concentration of 75 μmol l^{-1}. Again both learning and memory occurred (ANOVA $F_{(10,2)}$=6.6139, P<0.01; sessions 2 and 3 significantly different from session 1, P<0.05 and P<0.01, respectively).

We next asked whether a lower concentration of H_2S would similarly affect a snail's ability to learn and form memory. We therefore repeated the conditioning experiments in 50 and 75 μmol l^{-1} H_2S-hypoxic pondwater respectively (Fig. 3). In contrast to the results obtained with 100 μmol l^{-1} H_2S-hypoxic pond water, we found that snails exposed to either 50 (Fig. 3A) or 75 μmol l^{-1} H_2S (Fig. 3B) could both learn and form memory. That is, both the cohort exposed to 50 μmol l^{-1} and the cohort exposed to 75 μmol l^{-1} H_2S demonstrated learning (i.e. the last training session was significantly lower (P<0.01) than the first). Both groups also demonstrated memory [i.e. the

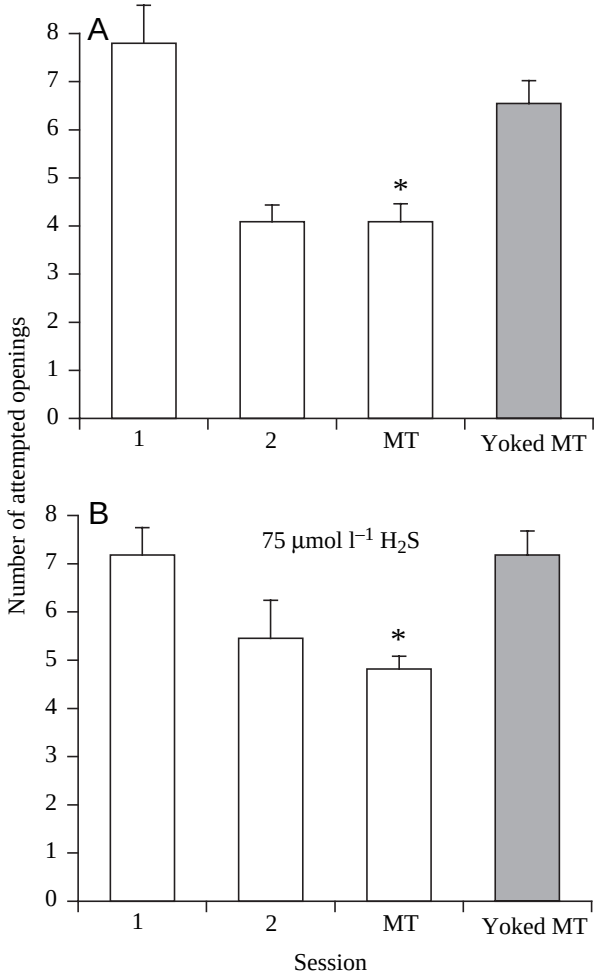

Fig. 4. Yoked control snails do not exhibit LTM. Snails were randomly matched to snails in the operantly conditioned group. These yoked control snails received the same number of stimuli (but in a non-contingent fashion) in sessions 1 and 2. In the third session (MT) 24 h later, snails in the yoked group now received a reinforcing stimulus each time they attempted to open their pneumostome. (A) Yoked controls under standard hypoxic conditions. The yoked group (N=11) had significantly more attempted openings in the memory test (MT) session than did the trained group (N=11) (ANOVA $F_{(21,5)}$=50.1381, P<0.01). (B) Yoked controls challenged with 75 μmol l^{-1} H_2S hypoxic conditions. Yoked snails (N=11) had significantly more attempted openings in the MT session as compared to the trained group (N=11) (ANOVA $F_{(21,5)}$=15.4653, P<0.01).

memory test session (MT) was significantly lower (P<0.01) than the session 1 and was not significantly greater than session 2]. To show that these changes in behaviour were *bona fide* examples of associative learning we performed yoked control experiments. The yoked control snails did not show any LTM formation in either group (i.e. the standard hypoxic (N=11), and hypoxic 75 μmol l^{-1} H_2S (N=11) group; Fig. 4).

To determine whether there was a diminished capability to learn and form memory in the H_2S challenged snails we gave each individual snail a 'grade' (see Materials and methods) and then determined if there were significant differences in the number of A grades (etc.) between the different cohorts (standard hypoxic; 'more intense-hypoxic challenge'; 50 μmol l^{-1} H_2S-hypoxic; 75 μmol l^{-1} H_2S-hypoxic; and 100 μmol l^{-1} H_2S-hypoxic, respectively, Fig. 5). As the concentration of H_2S is increased, the percentage of snails with 'A' grades decreases and more received an 'F' grade, which would be consistent with the hypothesis of a dose-dependent deleterious effect of the H_2S on the snails' ability to learn. A χ^2-statistical analysis was performed to determine if the snails trained under standard conditions and H_2S conditions showed a significantly different number of A and F grades (i.e. P<0.05). In this test the grades (A grades and F grades) of the snails trained under the standard conditions were used as the expected frequencies, and the snails trained in the H_2S and the 'more intense hypoxic challenge' were used as the observed frequencies to compare the two (such as in a placebo group *vs* a treatment group). There was no significant difference in the frequency of A grades and F grades between the standard hypoxic condition and 50 μmol l^{-1} H_2S-hypoxic group (P=0.081), but there was a significant difference between the standard hypoxic condition and 75 μmol l^{-1} H_2S-hypoxic group (P<0.01). Not surprisingly there was also a significant difference between the standard hypoxic group and the 100 μmol l^{-1} H_2S group (P<0.01). Thus as the concentration of H_2S in the hypoxic pondwater increases there is an increase in the frequency of snails that receive a failing grade. Finally, when we performed this analysis on the snails given the 'more intense-hypoxic challenge' we found that statistically they were the best learners and had the best memory. That is, they received statistically more A grades and fewer F grades than any of the other groups (P<0.01 when compared to the standard hypoxic group).

The finding that relatively low levels of H_2S in pondwater significantly diminished the ability of snails to learn prompted us to hypothesize that H_2S exposure would interfere with the memory consolidation process. Memory consolidation can be interfered with by a 1 h-cooling period (to 4°C) if it is applied within 10 min after the last training session (Smyth et al., 2002; Sangha et al., 2003a). We therefore exposed snails trained in standard hypoxic conditions to H_2S for 1 h immediately (within 30 s) after session 2. Following the 1 h exposure to H_2S the snails were returned to their home aquaria (Fig. 6). When we tested memory retention on the following day (MT) we found that snails had memory. That is, the number of attempted openings in MT was significantly different from the number of attempted openings in session 1 and was not significantly greater than the number of attempted openings in session 2. Thus, the memory consolidation process was not impeded by exposure to H_2S during the consolidation period.

Discussion

We hypothesized that: (1) relatively low levels of H_2S in pondwater would have significant effects on aerial respiratory behaviour in *Lymnaea*, and (2) the snails' ability to learn would be significantly impaired. We further hypothesized that exposure to H_2S in the immediate period following the acquisition of learning (i.e. the memory consolidation period) would prevent memory formation. Our data are consistent with our first two hypotheses but do not support the third. That is, exposure to H_2S: (1) significantly increases aerial respiration, (2) significantly diminishes the capacity to learn and remember, and (3) does not prevent memory consolidation or its accessibility if snails are exposed to H_2S *after* the acquisition of a new learned behaviour.

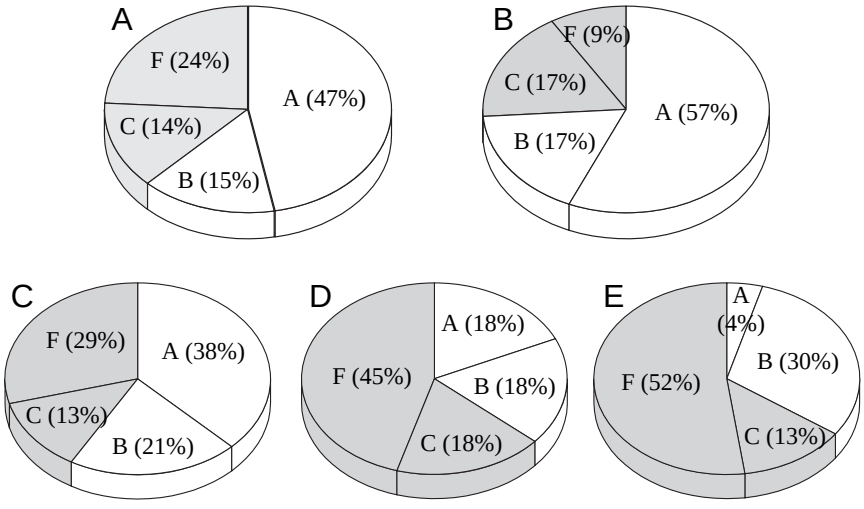

Fig. 5. Snail learning 'grade distributions'. Snails were given grades based on their individual performance. Grades were calculated as follows: a 50% reduction or greater is an A, a B is a reduction of 35–49.99%, a C is a 20–34.99% reduction, and an F is a reduction of less than 20%. (A) Grade distributions observed to occur under standard hypoxic conditions (N=2301). (B) Distribution of grades for snails presented with the 'more intense hypoxic challenge' (N=23). These snails showed a statistically greater number of A grades and fewer F grades than controls (P=0.0007). (C) Grade distributions seen for snails trained in the hypoxia + 50 μmol l^{-1} H_2S condition (N=24). (D) Distributions for snails trained in the hypoxia + 75 μmol l^{-1} H_2S (N=11). (E) Distributions for training in hypoxia + 100 μmol l^{-1} H_2S condition (N=23).

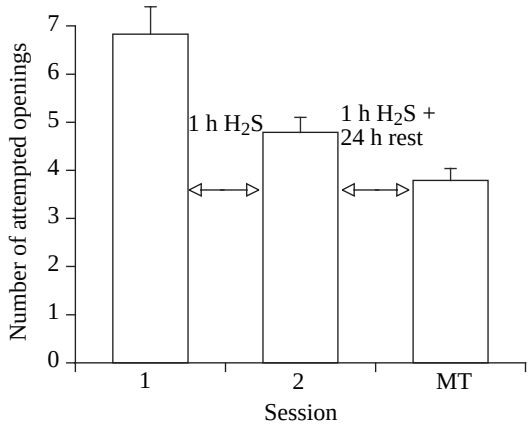

Fig. 6. The effect of H_2S on memory consolidation. Exposure to 100 μmol l^{-1} H_2S for 1 h immediately following the second training session had no effect on the ability of the animals to show memory 24 h later (N=24). Learning was shown since the number of attempted openings in session 2 was significantly less than session 1 (ANOVA $F_{(23,1)}$=8.6197, P<0.01). Memory was demonstrated since MT was significantly less than session 1 (ANOVA $F_{(23,1)}$=21.1944, P<0.01), but not significantly more than session 2 (ANOVA $F_{(23,1)}$=6.5714, P=0.0174).

Typically, in eumoxic conditions snails rely on cutaneous respiration to satisfy their respiratory requirements and perform aerial respiration only intermittently (Taylor and Lukowiak, 2001; Taylor et al., 2003). When snails encounter a hypoxic environment there is a significant increase in aerial respiratory behaviour. In other words, in a hypoxic environment cutaneous respiration is insufficient to meet the respiratory needs of the snail, and thus aerial respiration must be increased to satisfy the snails' oxygen requirements. We found that exposure to H_2S (100 μmol l^{-1}) in the hypoxic environment significantly and reversibly increased aerial respiratory behaviour, while having no effect on the oxygen content of the water. A similar increase in respiratory drive occurred when snails were maintained in a hypoxic environment where they were unable to perform aerial respiratory behaviour for a period (i.e. the 'more intense-hypoxic challenge') as shown here and previously (McComb et al., 2002). In that situation, snails develop an oxygen debt and significantly increase aerial respiratory behaviour to 'pay back' this debt. There are a number of possible explanations for the increase in aerial respiratory drive seen with the H_2S-challenge: (1) H_2S interferes with cutaneous respiration; (2) H_2S increases the O_2 requirements of the snail; (3) H_2S has a direct stimulatory effect on the respiratory CPG. At present we cannot distinguish between these possibilities; however, with the *in vitro* semi-intact preparation (Inoue et al., 1996; McComb et al., 2003) it may be possible to determine directly whether H_2S alters CPG activity. Such experiments will be initiated shortly.

Because the increase in aerial respiratory behaviour to the H_2S-challenge was reversible, any alteration in the efficiency of cutaneous respiration that could be attributed to the H_2S-challenge has to be short lived (i.e. <1 h). Likewise, if the H_2S-challenge causes an increase in the snails' O_2 requirements this increase would also have to be relatively short lived. We have not determined how long the increase in respiratory behaviour can be maintained in the H_2S-environment. Nor have we attempted to determine how long *Lymnaea* can remain viable in this concentration of H_2S. For example, will much longer H_2S exposure times lead to irreversible changes in aerial respiratory behaviour? These experiments will also be initiated in the future. Since CPG activity that drives aerial respiratory behaviour is easily modifiable (Taylor and Lukowiak, 2000) we presently favor the hypothesis that H_2S has direct effects on CPG output. That is, we expect to find that H_2S directly alters the activity of the CPG neurons so that rhythmogenesis is increased, as it is following 'more intense-hypoxic challenge', thus driving an increase in aerial respiratory behaviour.

We also hypothesized that an H_2S-challenge would affect the ability of *Lymnaea* to associatively learn. Our data show that there is a concentration-dependent effect of H_2S on the acquisition of operant conditioning, a form of associative learning. As the concentration of H_2S was increased from 50 μmol l^{-1} to 100 μmol l^{-1} there was a corresponding decrement in the snails' ability to acquire learning. At the lowest concentration tested here (50 μmol l^{-1}) the ability of the cohort of snails to acquire learning was not any different from the control cohort. However, at 75 μmol l^{-1} H_2S there was a significant decrease in the ability of the cohort of snails to acquire learning and at 100 μmol l^{-1} H_2S the cohort was incapable of learning. When we further analyzed each individual of the various cohorts the effects of the H_2S-challenge became even more apparent. We found that only 4% of snails challenged with the 100 μmol l^{-1} H_2S obtained a mark of A, whilst over 10 times that number received an A in the control standard hypoxic environment or the group following 'more intense-hypoxic challenge'. At the other end of the spectrum in the standard hypoxic situation, approximately 20% of snails received an F grade, whilst the majority (52%) of snails challenged with 100 μmol l^{-1} H_2S received an F grade. Similarly, examining the marks of individual snails challenged with 75 μmol l^{-1} H_2S we found that only 18% received an A grade, whilst 45% received an F. Again, these marks are indicative of a detrimental effect of 75 μmol l^{-1} H_2S on learning ability. We do not believe that the impairment in learning is caused by an increased need for aerial respiration as snails that receive the 'more intense-hypoxic challenge' had the most A grades and fewest F grades. That is, snails challenged with a procedure, that increases their respiratory needs, show no learning or memory deficits. Since yoked control snails did not show a change in aerial respiratory behaviour our data are consistent with the hypothesis that the detrimental effects of H_2S on learning and memory formation are the result of changes caused by H_2S on molecular processes in the neurons that are necessary for learning and memory.

Our data, however, did not support our final hypothesis, which was that an H_2S challenge would block the memory

consolidation process. Learning and memory are not a unitary process; rather they are separate but related processes (Dudai, 2003). Following the acquisition of a new behaviour (i.e. learning) there is a time period during which the learned behaviour is committed to memory (i.e. the consolidation process). When first acquired, memory is sensitive to disruption by external events. With the passage of time, however, storage becomes more permanent and less susceptible to disruption (White and Salinas, 1998). Brain injury, electroconvulsive shock, cooling and protein synthesis inhibitors can disturb memory, and even new learning if applied during the consolidation period (McGaugh, 1999; 2000).

In order to form a long-lasting memory (>5 h) in *Lymnaea*, as in all other animals, altered gene activity and new protein synthesis are required (Dudai, 2002). Thus, in *Lymnaea* the application of a transcription or translation inhibitor (Actinomycin D and Anisomycin, respectively) or quickly cooling the snail for 1 h at 4°C immediately after the last training session, prevents the formation of LTM (Sangha et al., 2003a,b). We therefore exposed snails to 100 μmol l^{-1} H_2S for 1 h immediately after the last operant conditioning training session. This procedure did not interfere with the formation of memory. That is, these snails still had the capability of consolidating the new learned behaviour into a memory that was not different from control. Thus, 100 μmol l^{-1} H_2S exposure, which blocked learning, did not block the consolidation processes that underlies the formation of memory. We did not test whether a longer exposure to 100 μmol l^{-1} H_2S would block memory formation. We therefore conclude that in *Lymnaea* a 100 μmol l^{-1} H_2S challenge only affects the learning process and not the memory consolidation process. Similar findings were also reported by Partlo et al. (2001), when they tested rats after exposure to H_2S. Thus an H_2S-challenge alters both declarative learning (spatial learning) in rats and non-declarative memory (operant conditioning) in snails.

In the rat, endogenous levels of H_2S (50–160 μmol l^{-1}) have been shown to facilitate the formation of LTP; however, at higher concentrations (320 and 640 μmol l^{-1}) this effect is no longer seen as population spikes and field EPSPs (excitatory post synaptic potentials) become suppressed (Abe and Kimura; 1996). Also lethal levels of sulfide have also been shown to be less than two times those of endogenous levels, indicating that the dose–response curve for H_2S is very steep (Warenycia et al; 1989). These phenomena may help to explain the results seen in our experiments. The lowest level of H_2S exposure used here (50 μmol l^{-1}) had little or no effect on aerial respiratory behaviour or learning ability. This may be due to the fact that there is not enough accumulation of H_2S, or it can be metabolized quickly enough so it does not reach a level where it can be detrimental to the learning process. However, at the higher concentrations used here (75 μmol l^{-1} and 100 μmol l^{-1}) the accumulation of H_2S may be sufficient to alter the synaptic interactions and/or endogenous membrane properties of the central pattern generator (CPG) in such a manner as to increase aerial respiratory behaviour and have a negative influence on the ability of the CPG to undergo the changes in neuronal activity that constitute the neural substrates of learning. It appears, for example, that there is an optimal range of RPeD1 (one of the three CPG neurons) activity within the respiratory CPG in *Lymnaea* that is conducive for optimizing aerial respiratory behaviour (McComb et al., 2003). Any increase or decrease in RPeD1's activity outside its optimal range has detrimental affects on the ability of this neural network to produce a respiratory rhythm.

Higher levels of H_2S have been hypothesized to cause the 'metabolic intoxication' seen in the mammalian brain (Wang, 2002). A similar 'intoxication' may be occurring in the CNS of *Lymnaea*, inhibiting the ability of the snail to acquire a learned response. In any case we have shown here that our *Lymnaea* model system can be used to study the effects of toxic gas on the ability to learn and form memory. Because we have shown that learning and memory formation require the soma of RPeD1, one of the three CPG neurons, we may be able to specify how H_2S alters learning and memory ability at the single neuron level.

We would like to thank Dr Kevin Cummings for his help with the oxygen content recordings, and the Canadian Institutes of Health Research for support.

References

Abe, K. and Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* **16**, 1066-1071.

Dudai, Y. (2002). Molecular bases of long-term memories: a question of persistence. *Curr. Opin. Neurobiol.* **12**, 211-216.

Dudai, Y. (2003). Neurobiology: Caught in the act. *Nature* **424**, 377-378.

Eto, K. and Kimura, H. (2002). The production of hydrogen sulfide is regulated by testosterone and S-adenosyl-L-methionine in mouse brain. *J. Neurochem.* **83**, 80-86.

Eto, K., Ogasawara, M., Umemura, K., Nagai, Y. and Kimura, H. (2002). Hydrogen sulfide is produced in response to neuronal excitation. *J. Neurosci.* **22**, 3386-3391.

Hessel, P. and Melenka, L. S. (1999). Health effects of acute hydrogen sulfide exposure in oil and gas workers. *Environ. Epidemiol. Toxicol.* **1**, 201-206.

Inoue, T., Takaski, K., Lukowiak, K. and Syed, N. (1996). Inhibition of the respiratory pattern-generating neurons by an identified wholebody withdrawal interneuron of *Lymnaea stagnalis*. *J. Exp. Biol.* **199**, 1887-1898.

Julian, D., Statile, J. L., Wohlgemuth, S. E. and Arp, A. J. (2002). Enzymatic hydrogen sulfide production in marine invertebrate tissues. *Comp. Biochem. Physiol.* **133A**, 105-115.

Kilburn, K. H. (1997). Exposure to reduced sulfur gases impairs neurobehavioral function. *South Med. J.* **90**, 997-1006.

Kimura, H. (2000). Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem. Biophys. Res. Commun.* **267**, 129-133.

Lukowiak, K., Adatia, A., Krygier, D. and Syed, N. (2000). Operant conditioning in *Lymnaea*: evidence for intermediate and long-term memory. *Learn. Mem.* **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., Haque, Z., Spencer, G., Varshney, N., Sangha, S. and Syed, N. (2003a). Long-term memory survives nerve injury and the subsequent regeneration process. *Learn. Mem.* **10**, 44-54.

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.

Lukowiak, K., Sangha, S., McComb, C., Varshay, N., Rosenegger, D.,

Sadamoto, H. and Scheibenstock, A. (2003b). Associative learning memory in the pond snail, *Lymnaea*. *J. Exp. Biol.* **206**, 2097-2103.

McComb, C., Sangha, S., Quadry, S., Yue, J., Scheibenstock, A. and Lukowiak, K. (2002). Context extinction and associative learning in *Lymnaea*. *Neurobiol. Learn. Mem.* **78**, 23-34.

McComb, C., Meems, R., Syed, N. and Lukowiak, K. (2003). Electrophysiological differences in the CPG aerial respiratory behavior between juvenile and adult *Lymnaea*. *J. Neurophysiol.* **90**, 983-992.

McGaugh, J. (1999). The preservation–consolidation hypothesis: Mueller and Pilzecker, 1900. *Brain Res. Bull.* **50**, 445-446.

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

Milby, T. H. and Baselt, R. C. (1999). Hydrogen sulfide poisoning: clarification of some controversial issues. *Amer. J. Ind. Med.* **35**, 192-195.

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

Partlo, L. A., Sainsbury, R. S. and Roth, S. H. (2001). Effects of repeated hydrogen sulphide (H_2S) exposure on learning and memory in the adult rat. *Neurotoxicol.* **22**, 177-189.

Reiffenstein, R. J., Hulbert, W. and Roth, S. H. (1992). Toxicology of hydrogen sulphide. *Ann. Rev. Pharmacol. Toxicol.* **32**, 109-134.

Roth, S. H. (1993). Hydrogen sulfide. In *Handbook of Hazardous Materials* (ed. M. Corn), pp. 367-376. New York: Academic Press Inc.

Roth, S. H. (1999). Toxicological effects of hydrogen sulfide in mammalian brain. *Environ. Epidemiol. Toxicol.* **1**, 241-248.

Roth, S. H. (in press). Toxicological and environmental impacts of hydrogen sulfide. In *Signal Transduction and the Gasotransmitters: NO, CO, and H_2S in Biology and Medicine*. Totowa, NJ: The Humana Press Inc.

Sangha, S., Scheibenstock, A., McComb, C. and Lukowiak, K. (2003a). Intermediate and long-term memories of associative learning are differentially affected by transcription *versus* translation blockers in *Lymnaea*. *J. Exp. Biol.* **206**, 1605-1613.

Sangha, S., Scheibenstock, A. and Lukowiak, K. (2003b). Reconsolidation of a long-term memory in *Lymnaea* requires new protein and RNA synthesis and the soma of RPeD1. *J. Neurosci.* **23**, 8034-8040.

Sangha, S., Scheibenstock, A., Morrow, R. and Lukowiak, K. (2003c). Extinction requires new RNA and protein synthesis and the soma of the cell RPeD1 in *Lymnaea stagnalis*. *J. Neurosci.* **23**, 9842-51.

Scheibenstock, A., Krygier, D., Haque, Z., Syed, N. and Lukowiak, K. (2002). The soma of RPeD1 must be present for long-term memory formation of associative learning in *Lymnaea*. *J. Neurophysiol.* **88**, 1584-1591.

Smyth, K., Sangha, S. and Lukowiak, K. (2002). Gone but not forgotten: The lingering effects of intermediate term memory on the persistence of LTM. *J. Exp. Biol.* **205**, 131-140.

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

Spencer, G. E., Kazmi, M. H., Syed, N. I. and Lukowiak, K. (2002). Changes in the activity of a CPG neuron after the reinforcement of an operantly conditioned behavior in *Lymnaea*. *J. Neurophysiol.* **88**, 1915-1923.

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

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

Taylor, B., Smyth, K., Burk, M., Lukowiak, K., Harris, M. and Remmers, J. (2003). Nitric oxide mediates metabolism as well as respiratory and cardiac responses to hypoxia in the snail *Lymnaea stagnalis*. *J. Exp. Zool.* **295A**, 37-46.

Taylor, B. E. and Lukowiak, K. (2000). The respiratory central pattern generator of *Lymnaea*: a model, measured and malleable. *Resp. Physiol.* **122**, 197-207.

Wang, R. (2002). Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J.* **16**, 1792-1798.

Warenycia, M. W., Goodwin, L. R., Benishin, C. G., Reiffenstein, R. J., Francom, D. M., Taylor, J. D. and Dieken, F. P. (1989). Acute hydrogen sulfide poisoning: demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. *Biochem. Pharmacol.* **38**, 973-981.

White, N. M. and Salinas, J. A. (1998). Pharmacological approaches to the study of learning and memory. In *Neurobiology of Learning and Memory* (ed. J. Martinez and R. Kesner), pp. 143-176. San Diego: Academic Press.