The Journal of Experimental Biology 214, 2118-2124

doi:10.1242/jeb.054635

RESEARCH ARTICLE

Low external environmental calcium levels prevent forgetting in *Lymnaea*

Bogdan Knezevic, Sarah Dalesman, Vikram Karnik, Jovita Byzitter and Ken Lukowiak*

Hotchkiss Brain Institute, University of Calgary, HSC 2104, 3330 Hospital Drive NW, Calgary, AB, Canada, T2N 4N1

*Author for correspondence (lukowiak@ucalgary.ca)

Accepted 15 March 2011

SUMMARY

Forgetting may allow an animal to react more appropriately to current conditions, rather than continuing to exhibit a previously learned, possibly maladaptive behaviour based on previous experience. One theory is that forgetting is an active process, whereby the previously learnt response is replaced by new learning that interferes with the older memory. Hence, we hypothesized that an appropriately timed environmental stressor that blocks long-term memory (LTM) formation would also block forgetting. *Lymnaea stagnalis* (L.) is a freshwater snail, which requires environmental calcium of at least 20 mg l^{-1} to meet its requirements. Low environmental Ca^{2+} (i.e. 20 mg l^{-1}) in their environment acts as a stressor, and prevents LTM formation. Here, we asked whether a low Ca^{2+} environment would also prevent forgetting, concordant with the retrograde interference model of Jenkins and Dallenbach. Snails were operantly conditioned to reduce aerial respiration in hypoxia. When maintained in standard conditions (80 mg l^{-1} Ca^{2+}), snails demonstrated LTM following training lasting 24 h, but not 72 h; however, when trained in standard conditions then exposed to a low Ca^{2+} environment (20 mg l^{-1}) immediately following training, they retained memory for at least 96 h, indicating that forgetting had been blocked. Thus, when exposed to low environmental Ca^{2+}, *Lymnaea* will fail to form new memories, but will also continue to retain information previously learned and remembered as the low calcium blocks forgetting.

Key words: *Lymnaea stagnalis*, long-term memory, environmental calcium, operant conditioning, forgetting.

INTRODUCTION

Forgetting has been hypothesized to occur either as a result of a passive decay process or because of active interference from other cognitive processes (Wixted, 2004; Sangha et al., 2005). The two dominant theories of forgetting can be summarized as: (1) forgetting is caused by a decay of the memory trace (Gates, 1930), e.g. the deterioration of the molecular underpinnings of the trace due to natural metabolic processes, or (2) forgetting is produced by interference from conflicting associations, e.g. retroactive interference, RI (i.e. learning and forming memory of something new) (Jenkins and Dallenbach, 1924). An active cognitive process that could cause forgetting would be if learning new information after a prior learning event and its subsequent consolidation into memory interfered with the 'older' memory. If this theory is correct, forgetting can be thought of as a process that ultimately depends on altered gene activity and new protein synthesis in neurons necessary for the original memory, and can be attributed to new memory formation causing a new memory to replace an older one, effectively 'removing' it (Sangha et al., 2005). A recent report using *Drosophila* (Shuai et al., 2010) shows that a small G-protein, Rac, plays a major role in forgetting. Up-regulation of Rac increases forgetting while its down-regulation increases memory persistence. However, this recent report did not examine the altered gene activity and new protein synthesis-dependent long-term memory (LTM) in *Drosophila*, but rather concentrated on an early memory that typically only persists for a few hours. Here, we hypothesized that in *Lymnaea* forgetting is an active process involving the acquisition of a newer memory that interferes with the older memory. Thus, if we can block new LTM formation using a commonly occurring environmental stressor, we will prevent forgetting.

Calcium is a crucial element for all pond water species, including freshwater molluscs. For calciphilic species, including the great pond snail, *Lymnaea stagnalis*, environmental calcium is a major factor limiting distribution, with populations absent from environments containing less than 20 mg l^{-1} Ca^{2+} (Boycott, 1936; Macan, 1977; Briers, 2003). *Lymnaea* relies on dissolved calcium in the water to provide 80% of its calcium requirements, with the remainder coming from food sources (Van Der Borght and Van Puymbroek, 1966), and whilst *Lymnaea* populations are able to survive in 20 mg l^{-1} Ca^{2+}, they are unable to demonstrate shell thickening, which provides protection from predators (Rundle et al., 2004). Where environmental calcium is limited (less than 50 mg l^{-1}), there are metabolic costs associated with Ca^{2+} uptake from the environment into the snail to maintain internal Ca^{2+} homeostasis, and these costs are greatly reduced at the 80 mg l^{-1} level (Greenaway, 1971). For example, snails maintained at low environmental Ca^{2+} levels (i.e. 20 mg l^{-1}) for 1 week exhibit increased cutaneous respiration and decreased motility (Dalesman and Lukowiak, 2010). More recently we have shown that even a 1 h exposure to the low Ca^{2+} environment (20 mg l^{-1}) prior to operant conditioning of aerial respiration is sufficient to block the ability of snails to form LTM (Dalesman et al., 2011). Fluctuations in the concentration of Ca^{2+} in freshwater systems are common. For example, there can be a 3- to 10-fold change in the environmental Ca^{2+} concentration over the period of a year (Macan, 1950; Williams, 1970; McKillop and Harrison, 1972) (S.D., unpublished data). Therefore, acute periods of low Ca^{2+} availability are likely to be common in natural populations.

We (Dalesman et al., 2011) recently found that operantly conditioning snails, using a one-trial training procedure (1TT)

(Martens et al., 2007) in low Ca^{2+} pond water (PW) prevents the formation of LTM whilst allowing intermediate-term memory (ITM) to be formed. ITM differs from LTM in *Lymnaea* in that ITM persists for up to 3 h and is only dependent on new protein synthesis, whereas LTM (lasting 24 h or longer) also requires gene transcription (Scheibenstock et al., 2002; Sangha et al., 2003b). If, as we postulated above, forgetting is due to an active process whereby a new learned and remembered behaviour interferes with and destroys the older memory, then blocking the formation of the new LTM would prevent this interference from occurring, and thus forgetting should be attenuated. Based on this interference model of forgetting, we predicted that placing snails in the low Ca^{2+} environment after they had been operantly conditioned in standard Ca^{2+} would prevent forgetting. The training procedure used in the experiments reported here typically results in a LTM that persists for 1 day. That is, forgetting occurs and memory is not observed at 72 h. We hypothesized that snails in the low Ca^{2+} (20 mg l^{-1}) environment would not form new memory, and thus forgetting would not occur. That is, LTM would be observed 72 h or longer after training.

MATERIALS AND METHODS

Adult *Lymnaea*, 25±1 mm spire height, were raised from stock originally obtained from Vrije Universeit in Amsterdam. This population originated from wild snails collected in the 1950s from canals in a polder located near Utrecht. Adult snails were reared in aquaria filled with de-chlorinated tap water ([Ca^{2+}], 60±5 mg l^{-1}), in the snail-rearing facility at the University of Calgary. Snails were transferred 1 week prior to experiments into oxygenated artificial PW (0.26 g l^{-1} Instant Ocean®, Spectrum Brands Inc., Madison, WI, USA) with additional calcium sulphate dihydrate added to make standard Ca^{2+} PW (i.e. 80 mg l^{-1}). Snails were maintained at room temperature (20±1°C) at a stocking density of 1 snail per litre and fed romaine lettuce *ad libitum*. Romaine lettuce has been used as a food source to successfully rear snails at this facility for several years, and although it contains a source of calcium that the snails would be able to utilize, the calcium content is fairly low (0.36 mg Ca^{2+} per gram of lettuce). Previous work has suggested that *Lymnaea* obtains the majority of its calcium requirements from the water (Van Der Borght and Van Puymbroek, 1966). Low Ca^{2+} for exposure pre- or post-training was made using artificial PW (0.26 g l^{-1}, Instant Ocean®) with additional calcium sulphate dihydrate to bring the Ca^{2+} level up to 20 mg l^{-1}.

Snails were held in standard Ca^{2+} conditions (80 mg l^{-1}) or low Ca^{2+} (20 mg l^{-1}) depending on the treatment group for 1 week prior to training. Operant conditioning to reduce aerial respiration in hypoxic conditions was carried out using a training procedure consisting of two 0.5 h training sessions separated by 1 h (Sangha et al., 2003b; Parvez et al., 2005). Briefly, 500 ml of PW (with the same [Ca^{2+}] in which snails were maintained immediately prior to training) was placed in a 1 l glass beaker. N_2 was then bubbled vigorously through the water for 20 min to make the water hypoxic (<5% O_2), followed by gentle bubbling during the training phase. Snails were placed in the beaker and allowed to acclimate for 10 min prior to training. Training session 1 (TR1) was then carried out for 30 min, in which the snail was gently prodded on the pneumostome using a wooden stick each time it attempted to open the pneumostome to perform aerial respiration. This resulted in the snail closing the pneumostome but not a full body withdrawal. The snail was then returned to its home aquarium in eumoxic PW for 1 h and then a further 30 min training session (TR2) was carried out using identical methods to TR1. Following TR2, snails were either returned to eumoxic conditions in the [Ca^{2+}] in which they had previously been maintained or moved to a new [Ca^{2+}], depending on the treatment group (see below). Snails were then tested for LTM formation 24, 72 or 96 h after TR2. The 0.5 h memory test was carried out in exactly the same way as the training session.

Treatment groups

LTM under the same training and testing Ca^{2+} conditions

We previously demonstrated that holding snails in low Ca^{2+} (20 mg l^{-1}) blocks LTM formation following a 1TT procedure (Dalesman et al., 2011). In this procedure, the snail is exposed to 25 μmol l^{-1} KCl contingent with pneumostome opening. We had not demonstrated that this blocking of LTM formation was also the case when snails were trained using our 'poking' method. Snails were held in either low (20 mg l^{-1}) or standard (80 mg l^{-1}) Ca^{2+} PW for 1 week prior to training. They were then trained and tested for LTM (24 h in both Ca^{2+} conditions and 72 h in standard calcium alone), as outlined above, in the same Ca^{2+} conditions in which they had been maintained prior to training.

LTM in changed Ca^{2+} conditions following training in standard Ca^{2+}

Snails were held for 1 week prior to training in standard Ca^{2+} PW (80 mg l^{-1}) in eumoxic aquaria. Both training and testing for LTM were carried out in standard Ca^{2+} PW, as outlined above; however, the Ca^{2+} environment in which snails were held in eumoxia between TR2 and testing for LTM was altered between treatment groups. To test whether the low Ca^{2+} environment between training and testing prevents forgetting, snails were held in low Ca^{2+} (20 mg l^{-1}) immediately following TR2 until testing (in standard calcium, i.e. 80 mg l^{-1} Ca^{2+}) for LTM at 72 and 96 h following TR2. We also carried out a series of controls for the effect of low Ca^{2+} following training: (1) snails were transferred into low Ca^{2+} for 24 h immediately following TR2, then returned to standard Ca^{2+} for 72 h prior to testing for LTM in standard Ca^{2+} 96 h following training; (2) snails were maintained in standard Ca^{2+} for 72 h immediately following TR2, then transferred into low Ca^{2+} for 24 h prior to testing for LTM in standard Ca^{2+}, 96 h following training; (3) snails were transferred into low Ca^{2+} for 72 h immediately following TR2 then held in standard Ca^{2+} for 24 h prior to testing for LTM in standard Ca^{2+}, 96 h following training.

LTM in changed Ca^{2+} conditions following training in low Ca^{2+}

To assess whether the effect of low Ca^{2+} on preventing forgetting was purely due to the snails being held in an alternative Ca^{2+} context between training and testing, we reversed the Ca^{2+} conditions they experienced during training and testing. Snails were held for 1 week prior to training in low Ca^{2+} PW (20 mg l^{-1}) in eumoxic aquaria. Both training and testing for LTM were carried out in low Ca^{2+} PW, as outlined above; however, the snails were held in standard (80 mg l^{-1}) Ca^{2+} immediately following TR2 until testing for LTM in low Ca^{2+} 96 h later.

Criteria for LTM

We operationally define memory in the following manner. Memory was considered to be present if the number of attempted pneumostome openings in a memory test session was significantly lower than that of TR1 and was not significantly higher than that of TR2 (Sangha et al., 2002).

Total breathing time

To control for the possibility that in the interval between TR2 and the memory test session when snails are in eumoxic conditions the snails in the low Ca^{2+} environment perform aerial respiration less often than those maintained in standard Ca^{2+} conditions, we performed the following experiment. Snails were trained in the standard Ca^{2+} environment and then were randomly assigned to two groups (N=24 for each group). One group was held in the standard Ca^{2+} environment whilst the other group was held in the low Ca^{2+} environment in eumoxia. We then measured the total breathing time (TBT) for 1 h in the eumoxic conditions in which the snails had been held for either 24 or 72 h following training.

Statistics

Data to test memory formation were analysed using repeated measures ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, USA), comparing the number of attempted pneumostome openings between the training sessions and test session. For all analyses, homogeneity of variance was confirmed using Mauchly's test for sphericity prior to analysis. Data for each 'testing' time period and calcium exposure condition were analysed separately, with training session (TR1 and TR2) and test session used for the within-subject comparison. Where overall significance was found, *post hoc* paired *t*-tests were used to assess whether the number of attempted pneumostome openings differed between TR1 and TR2 and between TR1 and the test session for LTM (asterisks on each figure indicate where the number of attempted pneumostome openings declines significantly relative to that in TR1). Data for TBT were analysed using a 2-way ANOVA in SPSS with calcium concentration in which snails were held following training (standard *vs* low) and time between training and testing for breathing time (24 *vs* 72 h) as factors.

RESULTS

LTM under the same training and testing Ca^{2+} conditions

In a previous study, we (Dalesman et al., 2011) examined the effects of a low Ca^{2+} environment (i.e. 20 mg l^{-1}) on the ability of *Lymnaea* to form LTM following a 1TT procedure and found that LTM formation was blocked. The training procedure used here, referred to as the 'standard training procedure', however, is different in that each time the snail attempted to open its pneumostome in the 0.5 h training session it received a gentle poke to the pneumostome area, which caused the snail to close the pneumostome. Thus, we first had to demonstrate, using the standard training procedure, that maintaining snails in the low Ca^{2+} environment for 1 week also blocked the formation of LTM relative to snails held in our standard (80 mg l^{-1}) Ca^{2+} PW. These data are presented in Fig. 1.

When snails were maintained, trained and tested in standard Ca^{2+} (80 mg l^{-1}) conditions, they demonstrated LTM 24 h ($F_{2,14}$=7.412, P=0.006; paired *t*-test TR1 *vs* test at 24 h: t=3.37, P=0.012), but not 72 h ($F_{2,18}$=6.481, P=0.008; paired *t*-test TR1 *vs* test at 72 h: P>0.05) following training (Fig. 1A). This was comparable to previous work, where our laboratory snails formed LTM lasting 24 h but not 48 h following two 0.5 h training sessions to reduce aerial respiration (Sangha et al., 2003b) and also following 1TT (Dalesman et al., 2011) in standard PW. However, snails that had been maintained, trained and tested in a low Ca^{2+} environment (20 mg l^{-1}) did not show LTM 24 h after the TR2 (Fig. 1B), in agreement with results from our alternative training procedure, 1TT (Dalesman et al., 2011). In the low Ca^{2+} environment, snails exhibited learning as the number of attempted pneumostome openings in TR2 was significantly fewer than in TR1 ($F_{2,22}$=6.53, P=0.006; paired *t*-test TR1 *vs* TR2: t=4.49, P=0.001). However, the criteria necessary to show that LTM was present were not met. That is, the number of attempted openings in the memory test was not significantly lower than in TR1 (paired *t*-test TR1 *vs* test at 24 h: t=0.96, P>0.05) and it was significantly greater than the number in TR2 (paired *t*-test TR2 *vs* test at 24 h: t=–3.38, P=0.006). Thus, we extended the previous findings (Dalesman et al., 2011) showing that the low Ca^{2+} environment also blocks LTM formation when the standard training procedure is used.

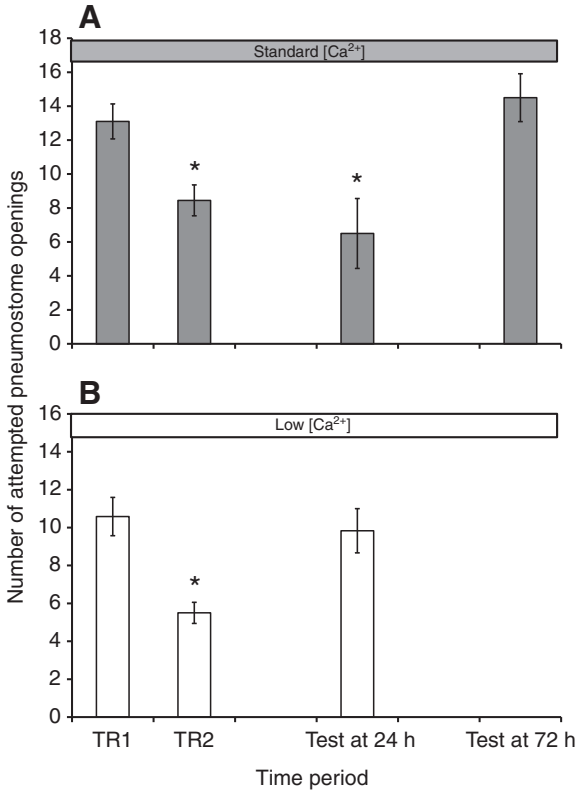

Fig. 1. Learning and memory formation under constant calcium conditions. The number of attempted pneumostome openings (means ± s.e.) in 30 min during training sessions (TR1 and TR2) and memory test session (Test) in: (A) standard Ca^{2+} (80 mg l^{-1}) and (B) low Ca^{2+} (20 mg l^{-1}) throughout the training and testing sessions. Column shading indicates conditions during training/testing (grey, standard Ca^{2+}; white, low Ca^{2+}); shading of the horizontal bar above the columns indicates conditions whilst snails were held in their eumoxic aquaria (grey, standard Ca^{2+}; white, low Ca^{2+}). *Number of pneumostome openings differs significantly from that in TR1.

LTM in changed Ca^{2+} conditions following training in standard Ca^{2+}

Having shown that with the standard training procedure we obtained a memory that persisted for 24 h but not 72 h in standard PW (80 mg l^{-1} Ca^{2+}), we were ready to test our hypothesis that forgetting would be prevented if snails were placed in the low Ca^{2+} environment following training, as this environment would block the formation of new LTM that would interfere with the older memory. A new naive cohort of snails (N=24) was trained in the standard Ca^{2+} environment and immediately following the end of TR2 was placed in the eumoxic low Ca^{2+} environment. These snails exhibited learning (i.e. number of openings in TR2 was significantly lower than in TR1). The snails were then randomly chosen to be tested for LTM either 72 or 96 h later in the standard Ca^{2+} environment. As can be seen (Fig. 2), memory was present both 72 h ($F_{2,22}$=4.70, P=0.020; paired *t*-test TR1 *vs* test at 72 h: t=2.25,

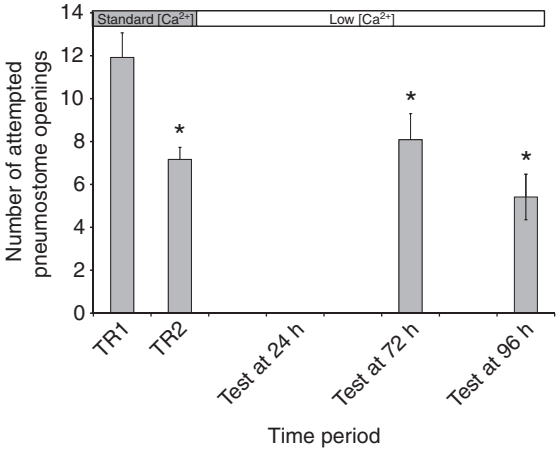

Fig. 2. Snails maintained in the low Ca^{2+} condition following training in the standard Ca^{2+} condition do not exhibit forgetting. The number of attempted pneumostome openings (means ± s.e.) in 30 min during training sessions (TR1 and TR2) and memory test sessions (Test) 72 and 96 h later. Column shading indicates conditions during training/testing (grey, standard Ca^{2+}). All training and testing sessions were performed in standard Ca^{2+}. Shading of the horizontal bar above the columns indicates conditions whilst snails were held in their eumoxic aquaria (grey, standard Ca^{2+}; white, low Ca^{2+}). *Number of pneumostome openings differs significantly from that in TR1.

P=0.046, N=12) and 96 h ($F_{2,22}$=8.81, P=0.002; paired t-test TR1 *vs* test at 96 h: t=2.89, 0.015, N=12) following training. That is, snails tested 72 or 96 h following TR2 made significantly fewer attempted pneumostome openings than they did in TR1, and did not make significantly more attempted openings than they did in TR2. This result is in marked contrast to the data presented in Fig. 1, where memory was only observed 24 h after TR2 and not 72 h after. The data presented in Fig. 2 are therefore consistent with our hypothesis that blocking new LTM formation would prevent forgetting of the initial memory.

We next performed a series of control experiments to demonstrate that the prevention of forgetting was due to the prevention of new memory formation and not to some 'side-effect' of placing snails in a low Ca^{2+} environment. These data are presented in Figs 3 and 4. As can be seen in Fig. 3A, training naive snails (N=11) in standard Ca^{2+} and then placing the snails in the low Ca^{2+} environment for 24 h before returning them to the standard Ca^{2+} environment for 72 h prior to testing did not prevent forgetting. That is, when snails were tested for LTM 96 h after TR2 they did not demonstrate LTM ($F_{2,20}$=6.916, P=0.005: paired t-test TR1 *vs* test at 96 h: t=–1.53, P>0.05). Thus, it was not just an exposure to the low Ca^{2+} environment for 24 h immediately following training that prevented forgetting. The second control experiment was similar to that described in Fig. 3A except that snails were held immediately following training in the standard Ca^{2+} environment for 72 h, then placed in the low Ca^{2+} environment 24 h before the memory test in the standard Ca^{2+} environment. As can be seen from Fig. 3B, LTM was not apparent when tested 96 h after training ($F_{2,20}$=7.62, P=0.004: paired t-test TR1 *vs* test at 96 h: t=–0.74, P>0.05). Thus, the experience of being in a low Ca^{2+} environment immediately prior to the test session does not significantly reduce the number of attempted pneumostome openings during the test. Therefore, placing snails in the low Ca^{2+} environment for 24 h either immediately after training or just before testing for memory 96 h after training is not sufficient to block forgetting.

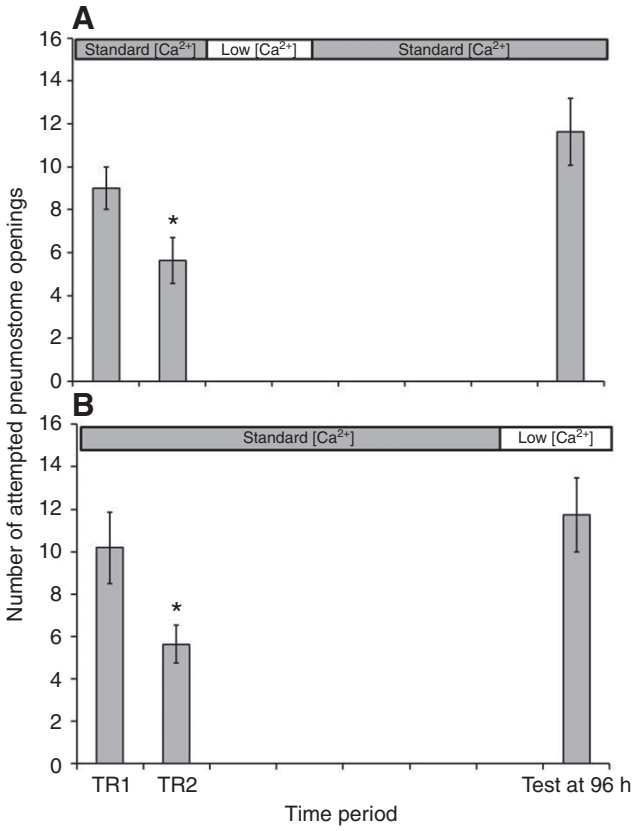

Fig. 3. A 24 h exposure to low Ca^{2+} just after training or just before recall does not prevent forgetting. The number of attempted pneumostome openings (means ± s.e.) in 30 min during training sessions (TR1 and TR2) and the memory test session (Test) 96 h later. Column shading indicates conditions during training/testing (grey, standard Ca^{2+}); shading of the horizontal bar above the columns indicates conditions whilst snails were held in their eumoxic aquaria (grey, standard Ca^{2+}; white, low Ca^{2+}). *Number of pneumostome openings differs significantly from that in TR1.

We then asked whether the changes in the prevention of forgetting by exposure to the low Ca^{2+} environment were due to a longer exposure time, i.e. snails had to be exposed to low Ca^{2+} for longer than 24 h following training (Fig. 4). Snails were trained in standard Ca^{2+} and then following training transferred to the low Ca^{2+} environment. However, what was different from the experiment performed in Fig. 3 is that the snails were maintained in low Ca^{2+} for 72 h and then transferred to standard Ca^{2+} for 24 h before memory was tested in the standard Ca^{2+}. As can be seen, LTM was not present (Fig. 4; $F_{2,22}$=11.74, P<0.001: paired t-test TR1 *vs* test at 96 h: t=–1.06, P>0.05). Thus, snails spending 24 h in standard Ca^{2+} before the memory test exhibited forgetting. That is, 24 h was sufficient time for the snails to learn and form new memory that interfered with the older memory and thus caused forgetting.

LTM in changed Ca^{2+} conditions following training in low Ca^{2+}

To be confident that the prevention of forgetting is due to maintaining snails after operant conditioning in the low Ca^{2+} environment rather than to a change of context, we 'reversed' the order in which snails experienced the low and standard Ca^{2+} environments and then tested for LTM. Thus, in Fig. 5 we trained a naive cohort of snails (N=11) in the low Ca^{2+} environment that they had been maintained in for 1 week prior to receiving operant conditioning training and then placed them immediately after

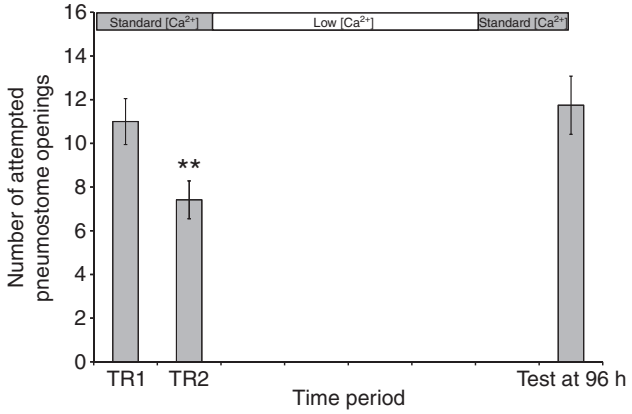

Fig. 4. A 24 h exposure to standard Ca^{2+} is sufficient to cause forgetting. This experiment is a control for the effect of 72 h exposure to low Ca^{2+} on forgetting. The number of attempted pneumostome openings (means ± s.e.) in 30 min during training sessions (TR1 and TR2) and the memory test session (Test) 96 h later. Column shading indicates conditions during training/testing (grey, standard Ca^{2+}); shading of the horizontal bar above the columns indicates conditions whilst snails were held in their eumoxic aquaria (grey, standard Ca^{2+}; white, low Ca^{2+}). *Number of pneumostome openings differs significantly from that of TR1.

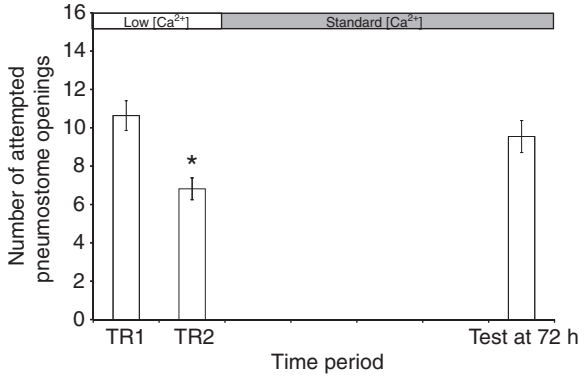

Fig. 5. Training in low Ca^{2+} and maintenance and testing in standard Ca^{2+} does not prevent forgetting. This experiment is essentially a control for change of context on forgetting. The number of attempted pneumostome openings (means ± s.e.) in 30 min during training sessions (TR1 and TR2) and the memory test session (test) 96 h later. Column shading indicates conditions during training/testing (white, low Ca^{2+}); shading of the horizontal bar above the columns indicates conditions whilst snails were held in their eumoxic aquaria (grey, standard Ca^{2+}; white, low Ca^{2+}). *Number of pneumostome openings differs significantly from TR1.

training into the standard Ca^{2+} environment for 72 h. We then tested for memory. While learning occurred in the low Ca^{2+} environment, LTM was not present 72 h after training ($F_{2,20}$=8.78, P=0.002: paired t-test TR1 *vs* test at 72 h: t=0.96, P>0.05). Thus, snails maintained in standard Ca^{2+} could forget a memory if it had formed, and lack of forgetting was not due to the snail experiencing a different context between TR2 and the test session.

TBT following training in standard PW

Previously, we obtained data in support of the hypothesis that forgetting is due to interfering events, by preventing snails from coming to the surface and performing aerial respiratory behaviour between training and testing (Sangha et al., 2003a; Sangha et al., 2005). We interpreted those data as indicating that snails were not able to learn and form new memory if they could not open their pneumostome during this period. It was thus possible that the low Ca^{2+} environment prevented forgetting by causing a decrease in the amount of aerial respiration (and thus less interference) that would occur under eumoxic conditions in the interval between training and the memory test. We therefore asked whether aerial respiration was altered following training in the low *vs* the standard [Ca^{2+}] eumoxic condition.

We found that there was no significant difference in TBT between snails held in eumoxic conditions in either low or standard Ca^{2+} PW for 24 or 72 h following training in standard PW (Fig. 6). Thus, snails in the low Ca^{2+} eumoxic environment performed aerial respiration to the same extent as those maintained in standard Ca^{2+}.

DISCUSSION

We hypothesized that forgetting of the conditioned behaviour (i.e. suppression of aerial respiration) is due to the conflicting association that occurs as a result of 'new learning and memory formation' of unreinforced aerial respiratory behaviour that happens in the eumoxic home aquaria in the interval between operant conditioning training and testing for memory. The data presented here support this hypothesis, demonstrating that: (1) the low Ca^{2+} environment prevented the formation of LTM following the standard operant conditioning training procedure; and (2) the same low Ca^{2+} environment prevented forgetting of a learned and remembered behaviour if snails were placed in it following operant conditioning training. Previously, we showed that a low Ca^{2+} environment blocked LTM formation following a 1TT (Dalesman et al., 2011), which is a different training procedure from the one used here. We used the fact that exposure to the low Ca^{2+} environment blocked the formation of LTM to test whether the low Ca^{2+} environment would also block forgetting. Thus, the data we obtained are consistent with the hypothesis that forgetting is an active process consisting of new learning and the formation of new memory that interferes with the old memory. We conclude, therefore, that if new memory cannot be formed, then forgetting does not happen.

However, the effects of the low Ca^{2+} environment on forgetting were not permanent. If snails were trained in the standard Ca^{2+} environment, placed in the low Ca^{2+} environment for 72 h, and then placed back in the standard Ca^{2+} environment for 24 h they exhibited forgetting. That is, the new learning and memory formation that

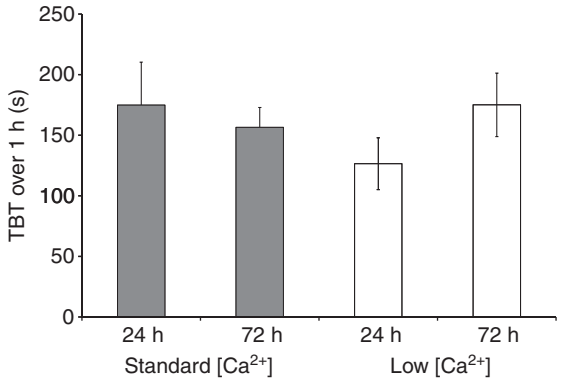

Fig. 6. There is no difference in aerial respiratory behaviour following training in low *vs* standard Ca^{2+}. Mean ± s.e. total breathing time (TBT) in eumoxia 24 or 72 h following training in standard Ca^{2+}. Snails were held and tested in either standard Ca^{2+} (grey bars) or low Ca^{2+} (white bars).

occurred in the normal Ca^{2+} environment for 24 h was sufficient to cause forgetting of the previous learned behaviour. Allowing snails the opportunity to form a 'new' memory in the standard Ca^{2+} environment whether it preceded or followed a 24 h exposure to the low Ca^{2+} environment also allowed forgetting to occur. These data are similar to those reported earlier where, after being kept submerged or cooled, forgetting occurred if the snails were maintained as they normally were for 24 h before testing (Sangha et al., 2003c; Sangha et al., 2005). In other words, when snails have the opportunity, even several days after the learning event, they can again form a 'new' memory that interferes with the 'older' memory and forgetting occurs.

We further found that snails exposed to the low Ca^{2+} environment for 1 week before training and during the two training sessions but then transferred to standard Ca^{2+} before testing for memory demonstrated learning (i.e. the number of openings in TR2 was significantly lower than that in TR1) but did not exhibit LTM when tested 72 h later. Thus, snails maintained and acclimated to the low Ca^{2+} environment were capable of performing both aerial respiration and associative learning but were not capable of exhibiting LTM 72 h later. This demonstrated that the lack of forgetting was not due to these processes (learning and forgetting) occurring in a different context (i.e. different calcium concentrations).

Assessing aerial respiratory behaviour following training showed that exposure to a low Ca^{2+} environment specifically blocked forgetting and was not due to some 'side-effect' on aerial respiratory behaviour. It could have been argued that snails maintained in low Ca^{2+} performed aerial respiration less often than snails maintained in the standard Ca^{2+} environment. If this were so, then the lack of forgetting could have been attributed to the fewer unreinforced occurrences of aerial respiration. In some respects this would have been equivalent to maintaining snails under a barrier to prevent aerial respiratory behaviour (Sangha et al., 2005). However, the data presented in Fig. 6 show that, following training, snails in the low Ca^{2+} eumoxic environment performed aerial respiration to the same extent as those maintained in standard Ca^{2+}. Thus, the prevention of forgetting by low environmental calcium was not due to snails performing aerial respiration less often in their eumoxic home aquaria between the training and memory testing sessions.

Thus, our new data presented here, and the previous data where after memory formation had occurred, cooling, preventing snails from performing aerial respiration or ablation of the soma of RPeD1 (the neuron necessary for LTM formation) all prevented forgetting (Sangha et al., 2003a; Sangha et al., 2003c; Sangha et al., 2005), are consistent with the hypothesis that forgetting is the result of retrograde interference. That is, some newly learned and remembered behaviour interferes with a 'similar' memory and causes that memory to be forgotten. However, placing snails in a low Ca^{2+} environment is both far easier and more ecologically relevant (see Introduction) than any of the above-mentioned techniques previously employed to prevent forgetting. Thus, environmental stressors such as a shift in external Ca^{2+} concentration can alter both memory formation and its persistence (i.e. blocking forgetting). For the snail, a low calcium environment could therefore result in an inability to form memory about food sources, conspecific interactions or predation threat during periods of low calcium availability. Equally, however, it could also prevent adaptive forgetting, whereby the snail retains irrelevant information. This could potentially prevent it from performing normal behaviours, such as blocking aerial respiration in hypoxia as shown here, when there is no reason to do so.

We do not currently understand at the causal neuronal or molecular level how exposure to the low Ca^{2+} environment prevented LTM formation (Dalesman et al., 2011). In our previous study, we suggested that snails detect low environmental Ca^{2+} possibly by sensory neurons located in the osphradium, and that the activity of these neurons alters the molecular machinery in RPeD1 such that the molecular steps necessary for memory consolidation to produce behavioural LTM cannot occur (Dalesman et al., 2011). Preventing new memory in this manner would ultimately prevent forgetting. How sensing low environmental Ca^{2+} alters the molecular machinery of neurons such as RPeD1 is not known. Genomic processes in neurons such as RPeD1 are quickly altered by input from sensory structures such as the osphradium (e.g. Il-Han et al., 2010) and alter behavioural plasticity in significant ways (e.g. preventing forgetting).

It appears, however, that a greater understanding of the molecular mechanisms that underlie forgetting has been elucidated in *Drosophila*. A G-protein named Rac appears to be critical for forgetting but not for learning to occur (Shuai et al., 2010). Using transgenic flies expressing as adults a dominant negative Rac in all mushroom body neurons (the neurons essential for memory), Shuai and colleagues showed that these flies had a more persistent memory than controls. If, however, they constructed flies expressing a constitutively active form of Rac in these neurons, the opposite happened – memory persisted significantly less than controls. Thus, they concluded that Rac acts as a 'rheostat' for memory. That is, inhibition of Rac activity suppresses forgetting whereas an increase in Rac activity accelerates forgetting. Shuai and colleagues concluded that active forgetting is due to Rac activation (Shuai et al., 2010). Rac is thought to act on actin/cytoskeletal dynamics that underlie structural changes at synaptic sites that are thought to be involved with memory stabilization. These *Drosophila* data suggest to us that the molecular mechanisms of forgetting may be different from those that underlie memory formation, even if both processes depend on altered gene activity and new protein synthesis (Sangha et al., 2005). That is, both memory formation and forgetting are active processes.

Historically, there have been two dominant theories of forgetting (Wixted, 2004): (1) it is caused by a decay of the memory trace (Gates, 1930), e.g. the deterioration of the molecular underpinnings of the trace due to natural metabolic processes, or (2) it is produced by interference from conflicting associations, e.g. RI (i.e. learning and forming memory of something new) (Jenkins and Dallenbach, 1924). The decay postulate holds that memory evaporates with time, whilst interference has at its core the idea that forgetting is the result of learning and forming memory anew. Data supporting the interference hypothesis come from experiments showing that there is greater memory retention when the interval between learning and memory testing is filled with non-learning or no new memory activity than when it is filled with learning (Jenkins and Dallenbach, 1924; Minami and Dallenbach, 1946; Walker et al., 2003; Sangha et al., 2005). Here, we directly tested the interference hypothesis by making use of the fact that snails exposed to a low Ca^{2+} environment, while capable of new learning, do not have the capacity to form new LTM. Thus, following training and memory formation in normal Ca^{2+}, snails maintained in low Ca^{2+} conditions for up to 96 h do not forget. In the behaviour study, an association occurs in the eumoxic home aquarium in the interval between training and testing for memory between aerial respiration and no reinforcement. These new associations will then be consolidated into a new memory, which will displace the earlier memory (i.e. bring about forgetting). Whether the new interfering memory in *Lymnaea* causes a molecular process to activate a G-protein

such as Rac is not known. We also cannot envisage an easy explanation of how the decay hypothesis could explain our new data. Thus, coupled with our previous findings (Sangha et al., 2003c; Sangha et al., 2005), we believe that the most parsimonious explanation of forgetting in *Lymnaea* is that it is an active process (i.e. altered gene activity and new protein synthesis) that involves learning and memory formation of a new behaviour that interferes with the older memory. The new learning and memory formation results in the destruction of the older memory (i.e. forgetting) (Sangha et al., 2005).

ACKNOWLEDGEMENTS

This study was funded by an Alberta Heritage Foundation for Medical Research (AHFMR) postdoctoral fellowship awarded to S.D., an AHFMR Summer studentship to V.K., a Hotchkiss Brain Institute Summer studentship to J.B. and NSERC funds K.L.

REFERENCES

Boycott, A. E. (1936). The habitats of fresh-water Mollusca in Britain. *J. Anim. Ecol.* **5**, 116-186.

Briers, R. A. (2003). Range size and environmental calcium requirements of British freshwater gastropods. *Glob. Ecol. Biogeogr.* **12**, 47-51.

Dalesman, S. and Lukowiak, K. (2011). Effect of acute exposure to low environmental calcium alters respiration and locomotion of *Lymnaea stagnalis* (L.). *J. Exp. Biol.* **213**, 1471-1476.

Dalesman, S., Braun, M. H. and Lukowiak, K. (2011). Low environmental calcium blocks long term memory formation in a pulmonate snail. *Neurobiol. Learn. Mem.* **95**, 393-403.

Gates, A. I. (1930). *Psychology for Students of Education*. New York: Macmillan.

Greenaway, P. (1971). Calcium regulation in the freshwater mollusc, *Limnaea stagnalis* (L.) (Gastropoda: Pulmonata). 1. The effect of internal and external calcium concentration. *J. Exp. Biol.* **54**, 199-214.

Il-Han, J., Janes, T. and Lukowiak, K. (2010). The role of serotonin in the enhancement of long-term memory resulting from predator detection in *Lymnaea*. *J. Exp. Biol.* **213**, 3603-3614.

Jenkins, J. and Dallenbach, K. (1924). Obliviscence during sleep and waking. *Am. Psychol.* **35**, 605-612.

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.

Macan, T. T. (1950). Ecology of fresh-water Mollusca in the English Lake District. *J. Anim. Ecol.* **19**, 124-146.

Macan, T. T. (1977). *A Key to the British Fresh- and Brackish-Water Gastropods with Note on their Ecology*. Ambleside: The Freshwater Biological Association.

Martens, K., Amarell, M., Parvez, K., Hittel, K., De Caigny, P., Ito, E. and Lukowiak, K. (2007). One-trial conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. *Neurobiol. Learn. Mem.* **88**, 232-242.

McKillop, W. and Harrison, A. (1972). Distribution of aquatic gastropods across the interface between the Canadian Shield and limestone formations. *Can. J. Zool.* **50**, 1433-1445.

Minami, H. and Dallenbach, K. (1946). The effect of activity upon learning and retention in the cockroach, *Periplaneta americana*. *Am. Psychol.* **59**, 1-58.

Parvez, K., Stewart, O., Sangha, S. and Lukowiak, K. (2005). Boosting intermediate-term into long-term memory. *J. Exp. Biol.* **208**, 1525-1536.

Rundle, S. D., Spicer, J. I., Coleman, R. A., Vosper, J. and Soane, J. (2004). Environmental calcium modifies induced defences in snails. *Proc. R. Soc. B* **271**, S67-S70.

Sangha, S., McComb, C., Scheibenstock, A., Johannes, C. and Lukowiak, K. (2002). The effects of continuous versus partial reinforcement schedules on associative learning, memory and extinction in *Lymnaea stagnalis*. *J. Exp. Biol.* **205**, 1171-1178.

Sangha, S., McComb, C. and Lukowiak, K. (2003a). Forgetting and the extension of memory in *Lymnaea*. *J. Exp. Biol.* **206**, 71-77.

Sangha, S., Scheibenstock, A., McComb, C. and Lukowiak, K. (2003b). 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., Morrow, R., Smyth, K., Cooke, R. and Lukowiak, K. (2003c). Cooling blocks ITM and LTM formation and preserves memory. *Neurbiol. Learn. Mem.* **80**, 130-139.

Sangha, S., Scheibenstock, A., Martens, K., Varshney, N., Cooke, R. and Lukowiak, K. (2005). Impairing forgetting by preventing new learning and memory. *Behav. Neurosci.* **119**, 787-796.

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.

Shuai, Y. C., Lu, B. Y., Hu, Y., Wang, L. Z., Sun, K. and Zhong, Y. (2010). Forgetting is regulated through rac activity in *Drosophila*. *Cell* **140**, 579-589.

Van Der Borght, O. and Van Puymbroek, S. (1966). Calcium metabolism in a freshwater mollusc: quantitative importance of water and food supply for calcium during growth. *Nature* **210**, 791-793.

Walker, M. P., Brakefield, T., Hobson, J. A. and Stickgold, R. (2003). Dissociable stages of human memory consolidation and reconsolidation. *Nature* **425**, 616-620.

Williams, N. V. (1970). Studies on aquatic pulmonate snails in central Africa 1. Field distribution in relation to water chemistry. *Malacologia* **10**, 153-164.

Wixted, J. T. (2004). The psychology and neuroscience of forgetting. *Annu. Rev. Psychol.* **55**, 235-269.