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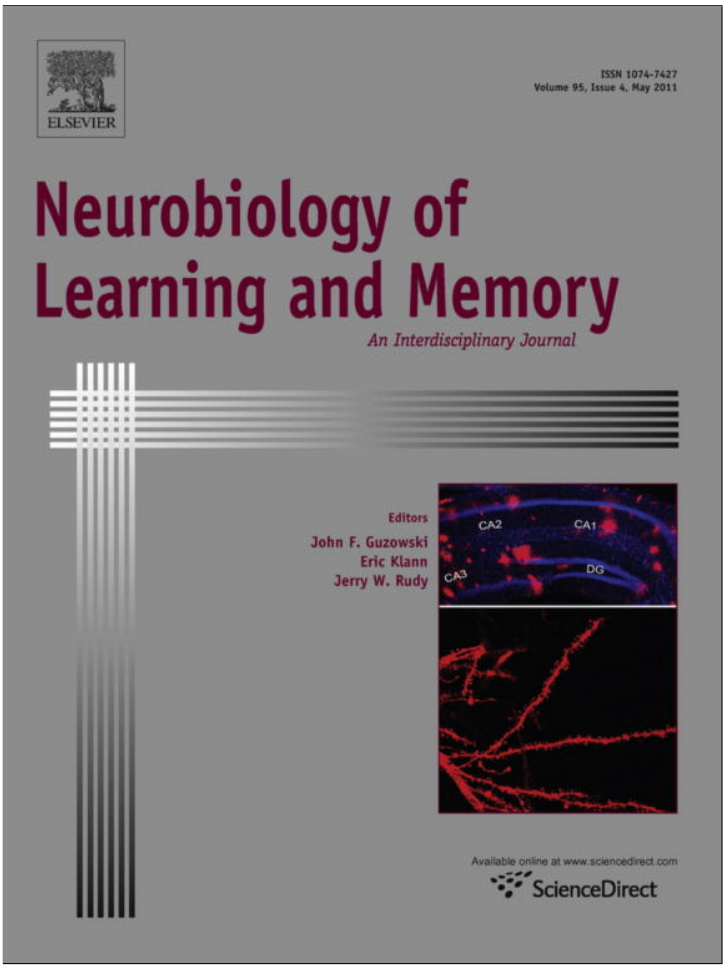

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Low environmental calcium blocks long-term memory formation in a freshwater pulmonate snail

Sarah Dalesman *, Marvin H. Braun, Ken Lukowiak

Hotchkiss Brain Institute, Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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ABSTRACT

The freshwater snail *Lymnaea stagnalis* (L.) is considered a calciphile and exhibits reduced growth and survival in environments containing less than 20 mg/l environmental calcium. Although it has no apparent effect on survival at 20 mg/l, reducing environmental calcium increases metabolic demand, and as such we consider that this level of calcium acts as a stressor on the snail. We exposed snails to acute periods of low environmental calcium and tested their ability to form intermediate-term memory (ITM) and long-term memory (LTM) following one trial operant conditioning (1TT) to reduce aerial respiratory activity in hypoxic conditions. We also assessed whether there were changes in the electrophysiological properties of a single neuron, right pedal dorsal 1 (RPeD1), which has been demonstrated to be necessary for LTM formation. Following training in high (80 mg/l) environmental calcium, *L. stagnalis* formed ITM and LTM lasting 24 h and demonstrated a significant reduction in all activity measured from RPeD1; however when snails were exposed to low (20 mg/l) environmental calcium they were able to form ITM but not LTM. Although no behavioral LTM was formed, a partial reduction in RPeD1 activtiy measured 24 h after training was observed, indicating a residual effect of training. The strong effect that environmental calcium concentration had on physiology and behavior in response to training to reduce aerial respiration in *L. stagnalis* suggests that it is an element of gastropod husbandry that needs to be carefully considered when studying other traits. This study also indicates that *L. stagnalis* found naturally in low calcium environments may be less able to adapt to novel stressors than populations found in harder waters.

1. Introduction

Memory formation is a dynamic process that can be modulated by stressful events occurring during, before or after the learning process. The effect that stressors have on memory formation varies greatly depending on the type of stress experienced, the level of stress applied and the timing of the stressful event relative to the learning process, and can either enhance or block memory formation (Shors, 2004). The freshwater snail, *Lymnaea stagnalis*, provides an ideal model species to study learning and memory. A three-neuron central pattern generator drives aerial respiration in this species (Syed, Bulloch, & Lukowiak, 1990; Syed, Ridgway, Lukowiak, & Bulloch, 1992), and this behavior can be operantly conditioned to result in long-term memory (LTM) formation to reduce aerial respiration in hypoxia (Lukowiak, Ringseis, Spencer, Wildering, & Syed, 1996). This memory formation is flexible and can be both enhanced and blocked using environmentally relevant stressors, the timing of which relative to the learning procedure is critical (Lukowiak et al., 2010). For example, cooling immediately following a learning procedure blocks both intermediate-term memory (ITM) and LTM, but has no effect if applied 10–15 min following the training procedure (Sangha, Morrow, Smyth, Cooke, & Lukowiak, 2003). Conversely, detection of a sympatric predator during the training procedure can enhance the formation of LTM (Orr, Hittel, & Lukowiak, 2009; Orr & Lukowiak, 2008).

L. stagnalis is considered a calciphile, normally requiring $\geqslant$ 20 mg/l environmental calcium to survive and prosper in natural populations (Boycott, 1936; Madsen, 1987; Young, 1975b). Where *L. stagnalis* populations exist in areas with lower levels of environmental calcium, abundance is greatly reduced and no large adults were found, suggesting poor growth and survival (McKillop & Harrison, 1972). Low calcium environments also result in shell thinning in mollusks, potentially making them easier prey and less resistant to damage (Boycott, 1936; Glass & Darby,

Abbreviations: LTM, long-term memory; ITM, intermediate-term memory; 1TT, one trial operant conditioning; RPeD1, right pedal dorsal 1; CPG, central pattern generator.

* Corresponding author. Address: Hotchkiss Brain Institute, Department of Physiology and Pharmacology, University of Calgary, Room 2104, Health Sciences Centre, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. Fax: +1 403 283 2700.

E-mail addresses: sarah.dalesman@ucalgary.ca, sjdalesman@hotmail.com (S. Dalesman), mhbraun@ucalgary.ca (M.H. Braun), lukowiak@ucalgary.ca (K. Lukowiak).

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2009; Lewis & Magnuson, 1999; Madsen, 1987; Young, 1975a; Zalizniak, Kefford, & Nugegoda, 2009). For example, *L. stagnalis* demonstrates induced shell thickening in the presence of predator cues when sufficient environmental calcium is available (90 mg/l), but is unable to alter shell growth to the same degree in a moderate (45 mg/l) calcium environment (Rundle, Spicer, Coleman, Vosper, & Soane, 2004). Considering this, we would predict that despite populations surviving at 20 mg/l environmental calcium, the animals are not in ideal conditions and may be experiencing stress.

Whilst the effects of long-term exposure to low calcium levels on morphology are fairly well understood, behavioral responses to low environmental calcium are less well studied in freshwater gastropods. Fluctuations in the calcium concentration in freshwater systems are common, and can represent 3 to 10-fold changes in environmental calcium concentration over the period of a year (Macan, 1950; McKillop & Harrison, 1972; Williams, 1970; Dalesman unpublished data). As such, we might expect a rapid behavioral response to changes in the external environmental calcium concentration in addition to long term developmental responses. Aquatic gastropods are able to maintain haemolymph calcium levels in a low calcium environment (Dewith, Kok, & Vanderschors, 1987; Greenaway, 1971; Grosell & Brix, 2009), even in the complete absence of environmental calcium for at least 10 days (Dewith, 1977). However, calcium uptake requires energy in low external calcium concentrations. Passive transport of calcium from the external media can occur above 20 mg/l. Below 20 mg/l environmental calcium concentration in the external environment is lower than the snails internal calcium concentration, so all calcium uptake requires energy (Greenaway, 1971). Following acute exposure to low environmental calcium (i.e., 1 week), cutaneous respiration is increased and motility is decreased at 20 mg/l relative to 80 mg/l, indicating an increased basal metabolic rate in 20 mg/l environmental calcium, and a reduction in energy available for locomotion (Dalesman & Lukowiak, 2010). There is also evidence that *L. stagnalis* can sense environmental calcium concentrations, orientating toward higher calcium environments when given a choice (Piggott & Dussart, 1995). Consequently, we hypothesize that *L. stagnalis* will respond rapidly to low calcium concentration as an environmental stressor, and that this stress will modulate memory formation.

Here we assessed the potential for acute exposure to low environmental calcium to modulate memory formation by *L. stagnalis*, testing both ITM and LTM at two environmental calcium concentrations, low (20 mg/l) and high (80 mg/l). When we are not testing for the effects of environmental calcium concentration our laboratory population is maintained in 80 mg/l environmental calcium. As 1 week exposure to low calcium was sufficient to alter respiration and locomotory activity (Dalesman & Lukowiak, 2010), we first tested whether this length of exposure can also alter memory formation. Secondly, we assessed whether the ability to form LTM is rapidly reversed following just 1 h exposure to the alternate calcium environment prior to and during training. In addition to testing memory formation, we tested whether changes occur in the underlying neurophysiological properties of the three-neuron central pattern generator (CPG) that drives aerial respiratory behavior (Lukowiak et al., 1996, 1998, 2008; Syed, Bulloch, & Lukowiak, 1992; Syed et al., 1990). Specifically, we tested for changes in one of the CPG neurons, right pedal dorsal 1 (RPeD1). This neuron has been demonstrated to be necessary for LTM following operant conditioning using our 'poking' method where the pneumostome is gently prodded when the snail attempts to open it (Scheibenstock, Krygier, Haque, Syed, & Lukowiak, 2002). However, the effect of one-trial conditioning on the electrophysiological properties of this neuron at differing external environmental calcium concentrations had not yet been determined.

2. Material and methods

2.1. Animal source and maintenance

Adult *L. stagnalis* (L.), 25 ± 1 mm spire height, were raised from stock 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, containing 60 ± 5 mg/l environmental calcium, in the snail rearing facility at the University of Calgary, and were transferred 1 week prior to experiments into oxygenated artificial pond water (0.26 g/l Instant Ocean®, Spectrum Brands Inc., USA), with additional calcium sulfate dehydrate added to make to low calcium (20 mg/l) or high calcium (80 mg/l) water depending on treatment group. The calcium concentrations used were selected as they are within the natural range experienced by *L. stagnalis* in field conditions (Boycott, 1936), and have been shown to result in variation in respiration and motility following acute exposure (Dalesman & Lukowiak, 2010). Snails were maintained at room temperature (20 ± 1 °C) at a stocking density of one snail per liter 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), and previous work has suggested that *L. stagnalis* obtains the majority of its calcium requirements from the water (Van Der Borght & Van Puymbroek, 1966).

2.2. Behavioral measurements

2.2.1. Breathing observation

L. stagnalis are bi-modal breathers, able to absorb oxygen directly from the water via cutaneous respiration and also to respire aerially using a rudimentary lung opened to the atmosphere via the pneumostome (respiratory orifice). In eumoxic conditions, the primary form of respiration is cutaneous, however if oxygen saturation in the water is diminished the snails will switch to using aerial respiration. Pneumostome opening is an easily observable and recordable behavior, and duration of time spent aerially respiring can be increased by making the water hypoxic (Lukowiak et al., 1996).

Breathing observations were carried out in the same way in all experiments. 500 ml of either high or low environmental calcium pond water was placed in a 1 l beaker and made hypoxic (PO_2 < 931 Pa) by bubbling N_2 vigorously through the water for 20 min before the introduction of snails. N_2 bubbling was continued throughout the observation period to maintain hypoxic conditions, though at a reduced rate so as not to disturb aerial breathing behavior. Snails were acclimated to the beaker for 10 min prior to observation, then we recorded the total time spent aerially respiring (i.e., time when the pneumostome is open to the atmosphere) over a period of 30 min. Pre-observations were carried out 24 h prior to training to provide a baseline aerial breathing rate for each snail, which were compared with post-training observations to assess whether learning and memory formation had taken place. Post-training observations were carried out either 3 h following training to assess whether intermediate-term memory (ITM) had been formed, or 24 h or longer to assess whether long-term memory (LTM) had been formed (Lukowiak, Adatia, Krygier, & Syed, 2000; Martens et al., 2007).

2.2.2. Training protocol

L. stagnalis can be trained to reduce pneumostome breathing time in hypoxic conditions, both by operant training where the

pneumostome is gently prodded each time it is opened (Lukowiak et al., 1996, 1998, 2000), and via a one-trial conditioning procedure (1TT) detailed below (Martens et al., 2007). In this study, we used the 1TT conditioning procedure to assess memory capabilities at different environmental calcium concentrations.

One-trial conditioning uses the pairing of a stressful stimulus, in this case a 25 mM KCl bath, with the contingent occurrence of opening the pneumostome, to train the snail to reduce this action (Martens et al., 2007). Training was carried out in all cases 24 h after initial baseline breathing observations were made. N_2 was vigorously bubbled through 500 ml of artificial pond water for 20 min to produce hypoxic conditions. In the 1TT training session the snails were placed into the hypoxic pond water and moved to a 25 mM KCl bath when the first pneumostome opening attempt was made. They were held for 30 s in the KCl bath, and then returned to eumoxic pond water conditions in their home aquaria. This was then followed by testing in hypoxic pond water for memory, either 3 h for ITM or $\geqslant$24 h for LTM, after the training procedure.

Yoked controls, during the first set of experiments, were carried out to assess whether the experience of the KCl bath had a general (i.e. non-specific sensitizing) effect of altering aerial respiratory behavior. Breathing observations were carried out 24 h before and also 3 h and 24 h following the yoked control procedure. The yoked controls were carried out under identical conditions to the training outlined above, except in this case exposure to the KCl bath occurred when the snail to which they were yoked (paired with) opened its pneumostome. That is, placement in the KCl bath was not contingent on the snail opening its pneumostome. Therefore the yoked control snail did not associate opening its pneumostome with the KCl stressor.

The snail was considered to have formed memory if the total breathing time in the post-training observation period was significantly reduced relative to the pre-training observation period, and also significantly lower than yoked controls. If the training procedure was the cause of the alteration in breathing, the yoked controls should not differ significantly between the pre-training and post-training observations.

2.2.3. Effect of calcium concentration on ITM and LTM

To test for the effect of exposure of *L. stagnalis* to different environmental calcium concentrations on memory formation, we transferred adult snails into aquaria containing artificial pond water with either low (20 mg/l) or high (80 mg/l) environmental calcium for 1 week prior to testing. Snails were then trained in the calcium concentration in which they had been held using the 1TT conditioning with 25 mM KCl as outlined above (Martens et al., 2007). Snails were returned to their eumoxic aquaria between pre-training observation and training, and again following training until they were tested for memory. We tested snails for ITM at 3 h and LTM at 24 h after the 1TT training session using the standard observation protocol outlined above. At the same time as we trained snails, we also carried out the yoked control procedure on snails that had been held at each calcium concentration. As memory was formed in the high (i.e. 80 mg/l) calcium group but not in the low (i.e. 20 mg/l) calcium group at 24 h, we carried out a further trial testing for memory at 48 h in high calcium.

A further experiment (i.e., a 1 h change of the calcium concentration) was carried out to assess whether the effect of calcium concentration on LTM at 24 h could be rapidly reversed. Here snails were again held at either high (80 mg/l) or low (20 mg/l) calcium concentration for 1 week prior to the experiment. Pre-training observations were carried out in the initial calcium concentration in which they had held, and they were then maintained in that calcium concentration up to 1 h before training. One hour before training they were transferred into the alternate new calcium environment, such that snails that had been held in high environmental calcium (80 mg/l) were moved into low environmental calcium (20 mg/l), and snails that had previously been held in low calcium were moved into high calcium. One-trial conditioning was then carried out in the new environmental calcium concentration. Following the 1TT in the 'new' environmental calcium concentration, snails were then transferred back into their initial calcium environment (i.e., that in which they had been held for the previous week). Twenty-four hours following the 1TT training session, snails were then tested in their initial calcium environment to assess whether they had formed LTM.

2.2.4. Data analysis for behavioral trials

Data were analyzed using repeated measures ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). ITM and LTM, following 1 week exposure to low or high calcium environments, were tested using repeated measures ANOVA, with observation period (pre-training vs. post-training) used as the within-subject factor, and calcium level the snails had been maintained at (low vs. high) and training procedure (trained vs. yoked) as between subject factors. LTM at 48 h in high calcium was tested separately using a paired *t*-test in SPSS. A repeated measures ANOVA was used to test for rapid reversibility of the effect of calcium concentration, with observation period (pre-training vs. post-training) used as the within-subject factor, and calcium level the snails experienced during training (low vs. high) as the between subject factor.

All repeated measures data were tested for equality of variance using Mauchly's test for sphericity. Post-hoc paired *t*-tests with the *P*-value corrected for multiple comparisons were used to assess where significant differences were found within-subject.

2.3. Electrophysiological measurements

2.3.1. Electrophysiological recording from RPeD1

Snails were exposed to either high (80 mg/l) or low (20 mg/l) environmental calcium for 1 week prior to electrophysiological data collection, as outlined above. On the first day a baseline breathing rate was measured, followed 24 h later by the 1TT procedure (i.e., the identical training procedure to that used for behavior measures). Similarly to behavioral tests for LTM, trained snails and yoked controls were used to assess electrophysiological activity at 24 h post-training, when LTM would be expected to have formed in snails following 1TT (Martens et al., 2007). Naïve snails were also assessed to test for effects of calcium concentration alone on electrophysiological activity.

Semi-intact preparations were used to measure RPeD1 activity, and were prepared following protocols previously developed in our laboratory (McComb, Rosenegger, Varshney, Kwok, & Lukowiak, 2005; Orr, El-Bekai, Lui, Watson, & Lukowiak, 2007; Orr & Lukowiak, 2008; Orr et al., 2009). The level of the bathing solution was such that the pneumostome was just at the surface, as submersion prevents pneumostome opening from occurring. Semi-intact preparations were rested for 20 min prior to impaling RPeD1 with a sharp glass microelectrode filled with saturated K_2SO_4 solution (tip resistances ranged from 20 to 75 MΩ), then given a further 15 min stabilization prior to collection of electrophysiological data, for a total time of 35 min between dissection and data collection. Intracellular signals were amplified using a Neurodata Instruments IR283 amplifier (New York, NY, USA) and displayed simultaneously on a Macintosh PowerLab/4SP (ADInstruments Inc, Colorado Springs, CO, US) and a Hitachi oscilloscope (Tokyo, Japan). Recordings were stored and analyzed via Chart 5 software (AD Instruments Inc., Colorado Springs, CO, US) using a 600 s trace for data analysis. Electrode balance was measured at the start and

end of each experiment, and if the resistance had changed by more than 5% the trace was subsequently discarded.

One of the measurements taken from the trace was the number of action potential bursts fired. Previous work in our lab on isolated neurons, isolated brains and semi-intact preparations have revealed that the RPeD1 bursts of action potentials (APs) which trigger pneumostome opening have particular properties. Therefore, a burst was defined as a period of sustained depolarization during which 2 to over 20 action potentials were fired. The burst was not considered over until membrane potential had returned to the resting value.

A measure of RPeD1 excitability was obtained by counting the number of action potentials elicited when the impaled cell was driven through a series of 10 depolarizing current steps, from 0.2 to 2.0 nA. Each step was 400 ms long, and the cell was allowed to recover for 300 ms between steps. The input resistance of RPeD1 was measured by driving the impaled cell through a series of 10 hyperpolarizing current steps from −0.2 to −2.0 nA (step lengths are the same as above), and calculating the resistance from the resulting current–voltage relationships according to Ohm's law ($R = V/I$). Pilot studies showed that these measurements of excitability and resistance were very stable and reproducible, with negligible changes between trials. For this reason, most experiments only utilized two or three replicates (separated by ~30 s) to make certain that the values were not changing.

2.3.2. Data analysis for electrophysiological recording

The number of action potential bursts recorded from RPeD1 in 600 s, the number of action potentials elicited in response to the 10 depolarizing current steps and input resistance were included in the analysis of electrophysiological data. Due to heterogeneity of variance between treatment groups, data were log-transformed ($X + 1\log_{10}$) prior to analysis. Log-transformed data were analyzed using a one-way ANOVA (SPSS 17.0). Comparisons were made between the following conditions: naïve in high environmental calcium ($N = 12$); naïve in low environmental calcium ($N = 10$); trained in high environmental calcium ($N = 9$); trained in low environmental calcium ($N = 9$); yoked high environmental calcium ($N = 12$); yoked low environmental calcium ($N = 9$). Student–Newman–Keuls tests were used to carry out pair-wise post-hoc comparisons where overall differences were found. The resting membrane potential was also compared among treatment groups using a one-way ANOVA in SPSS, with treatment group as the factor in the analysis.

3. Results

3.1. Behavioral results

We have previously demonstrated that low (20 mg/l) environmental calcium significantly affects respiration and locomotion in *L. stagnalis* (Dalesman & Lukowiak, 2010). We have also shown that the 1TT procedure using KCl as a stressor normally results in both ITM at 3 h and LTM at 24 h (Martens et al., 2007). Here we examined the effect of low environmental (20 mg/l) calcium, which is an ecologically relevant calcium concentration for our species, on the ability of *L. stagnalis* to form ITM and LTM.

First we examined how exposure to a high (80 mg/l) or low (20 mg/l) calcium environment for a period of 1 week affected both ITM, which is dependent on new protein synthesis alone, and LTM, which is dependent on both new protein synthesis and altered gene activity (Sangha, Scheibenstock, McComb, & Lukowiak,

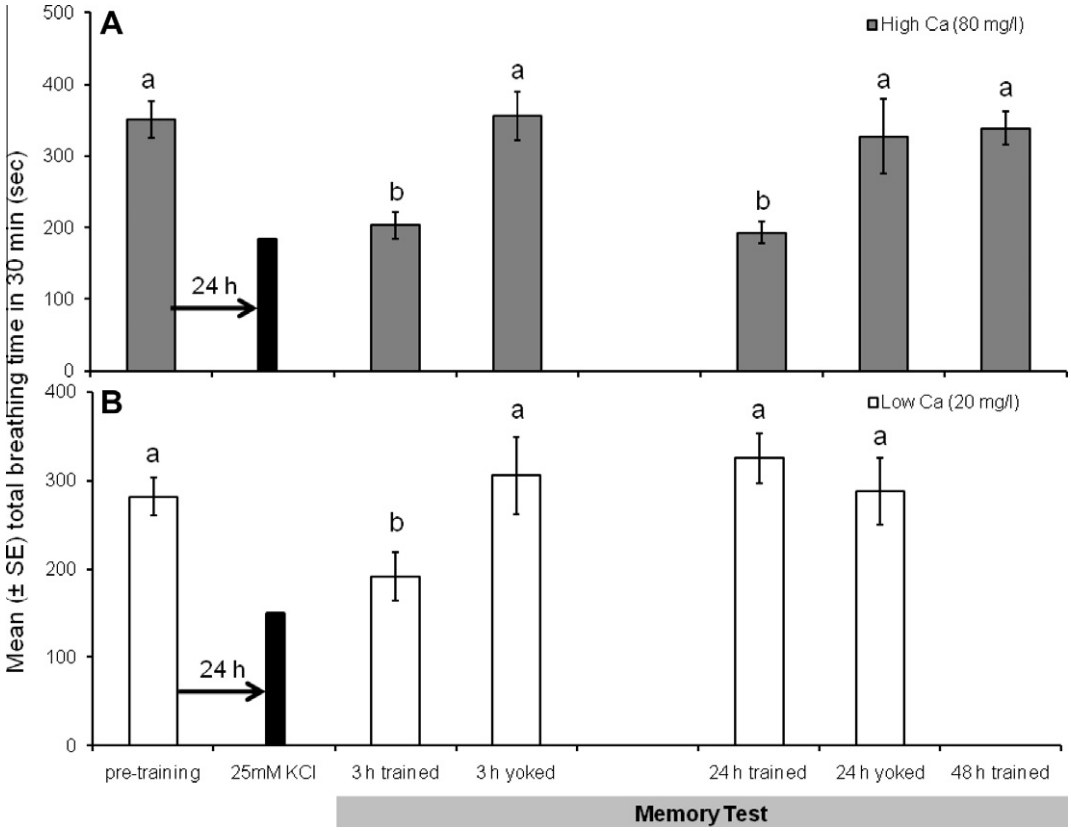

Fig. 1. Memory following one week exposure to high or low calcium environments. Mean total breathing time in high (shaded: 80 mg/l) and low (white: 20 mg/l) environmental calcium prior to and following one-trial conditioning (black bar). Different lower case lettering = significantly different (paired *t*-test corrected for multiple comparisons: $P < 0.016$).

2003). ITM, 3 h post-training, was formed in both the low (20 mg/l) and high (80 mg/l) calcium environments (Fig. 1A and B; 2-way interaction between pre- vs. post-training observation and training procedure, trained vs. yoked: $F_{1,61} = 11.06$, $P = 0.002$). Total breathing time was significantly lower at 3 h following the training procedure compared to the pre-training observation (high Ca: Fig. 1A; $t = 4.45$, $P < 0.001$, $N = 23$; low Ca: Fig. 1B; $t = 2.43$, $P = 0.024$, $N = 22$). There were no significant differences between pre-training and post-training observations for the yoked controls.

On the other hand, LTM formation differed depending on environmental calcium concentration. As Martens et al. (2007) found previously, LTM at 24 h was formed following the 1TT training procedure in the high (80 mg/l) calcium environment. However, following exposure to the low calcium (20 mg/l) environment for 1 week prior to and during the 1TT, the trained snails did not form LTM (Fig. 1A and B; 3-way interaction between time, pre- vs. post-training, training procedure, trained vs. yoked and calcium concentration, high vs. low: $F_{1,61} = 5.43$, $P = 0.023$). Total breathing time for trained snails decreased significantly between pre- and post-training in the high (80 mg/l) calcium environment (Fig. 1A; $t = 4.99$, $P < 0.001$, $N = 23$), but there was no significant difference between total breathing time pre- and post-training in the low (20 mg/l) calcium environment (Fig. 1B; $t = 1.29$, $P = 0.211$, $N = 22$). There were no differences between pre-training and post-training observations for the yoked controls tested for LTM. At 48 h following 1TT training there was no longer any evidence of memory formation in the high (80 mg/l) calcium environment (Fig. 1A), concurring with previous results where LTM in artificial pond water alone does not extend to 48 h (Martens et al., 2007).

Whilst we would consider 1 week exposure to either high or low calcium concentrations to be an 'acute' period, we have previously found changes in both aerial and cutaneous respiration following just 24 h exposure to a high (80 mg/l) or low (20 mg/l) calcium environment (Dalesman & Lukowiak, 2010). There is evidence that *L. stagnalis* is able to rapidly assess the calcium concentration in its environment from selection trials (Piggott & Dussart, 1995), and so we wanted to test whether behavior in the form of learning and its subsequent memory formation could be altered over shorter periods of time. Thus, we switched the calcium concentration in which the snails were maintained for 1 h prior to and during the training procedure, such that snails previously held in high calcium for 1 week experienced low calcium conditions for 1 h prior to and during 1TT training, and were then returned to high calcium conditions and vice versa. Both pre- and post-training observations were carried out in the calcium conditions in which the snail had been held for the week; only 1TT training occurred in the alternate conditions.

We found that the calcium conditions in which the snails received the 1TT significantly affected their ability to form LTM at 24 h. The snails held in a high calcium environment, but trained in low calcium, no longer formed LTM (Fig. 2A), whereas snails held in a low calcium environment but trained in high calcium conditions were now able to form LTM (Fig. 2B; 2-way interaction between pre- vs. post-training and calcium conditions in which the snails were trained, low vs. high: $F_{1,18} = 5.43$, $P = 0.032$). These data indicate that holding the snails for just 1 h prior to and during the 1TT training procedure in a specific calcium concentration was sufficient to determine whether LTM would be formed or not. That is, it appears that the environmental calcium concentration at the time of the 1TT is what determined whether or not LTM formed.

3.2. Electrophysiology results

The resting membrane potential of RPeD1 from snails tested did not differ significantly between treatment groups (one-way ANOVA: $P > 0.05$; naïve high Ca: −63.4 ± 1.1 mV; naïve low Ca: 60.8 ± 2.3 mV; yoked high Ca: 66.0 ± 1.1 mV; yoked low Ca: 63.7 ± 1.8 mV; trained high Ca: 63.8 ± 2.0 mV; train low Ca: 68.0 ± 1.4 mV).

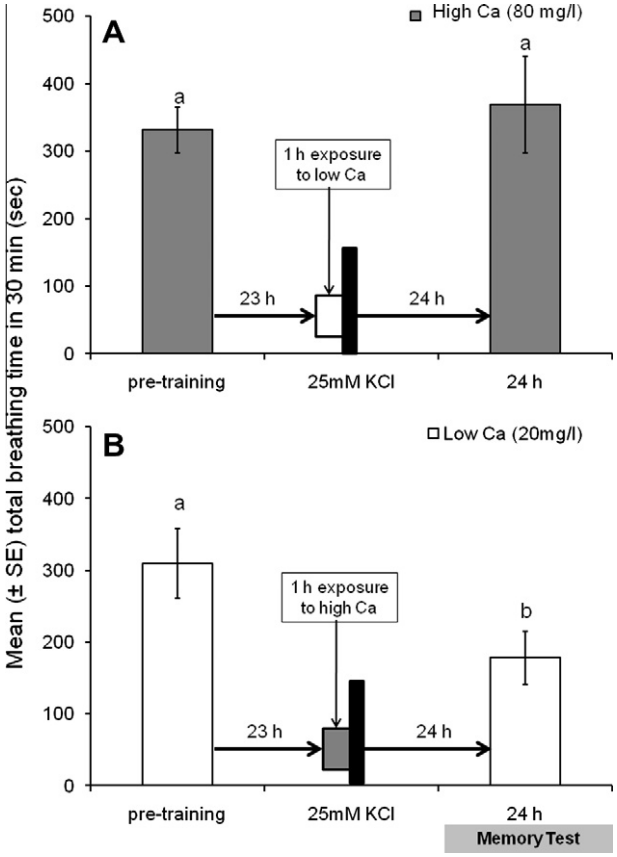

Fig. 2. Memory following 1 h exposure to high or low calcium environments. Mean total breathing time in high (shaded: 80 mg/l) and low (white: 20 mg/l) environmental calcium prior to and following one-trial conditioning (black bar). Snails were exposed to either high (shaded) or low (white) calcium for 1 h immediately prior to training. Different lower case lettering = significantly different (paired *t*-test corrected for multiple comparisons: $P < 0.016$).

Under normal circumstances, RPeD1 fires bursts of action potentials (Fig. 3B) that are correlated with the initiation of aerial respiratory activity, specifically opening of the pneumostome (McComb et al., 2005; Spencer, Kazmi, Syed, & Lukowiak, 2002; Syed et al., 1990). The behavioral data presented above (Figs. 1 and 2) show that in the high environmental calcium concentration LTM formed following the 1TT procedure. When we examined the activity of RPeD1 in semi-intact preparations 24 h after the 1TT procedure we found that all the electrophysiological parameters we measured were significantly altered in high environmental calcium concentrations, however in low environmental calcium conditions not all parameters were altered, and those that were showed a lesser reduction in activity.

We first examined the number of action potential bursts in RPeD1 from 1TT trained and naïve snails (Fig. 3). Grouped data showing the number of bursts recorded in RPeD1 24 h after the 1TT or the yoked control procedure in high and low environmental calcium conditions are shown (Fig. 3A). Representative data from three different preparations (naïve maintained in high calcium, 24 h trained in low environmental calcium and 24 h trained in high environmental calcium) are presented in Fig. 3B–D. Both training and the conditions under which they were trained significantly altered the burst activity (Fig. 3A; one-way ANOVA: $F_{5,55} = 9.16$, $P < 0.001$). Three points can be made from these data. The first is that there was no significant difference in the number of bursts seen in RPeD1 between naïve preparations and the yoked control

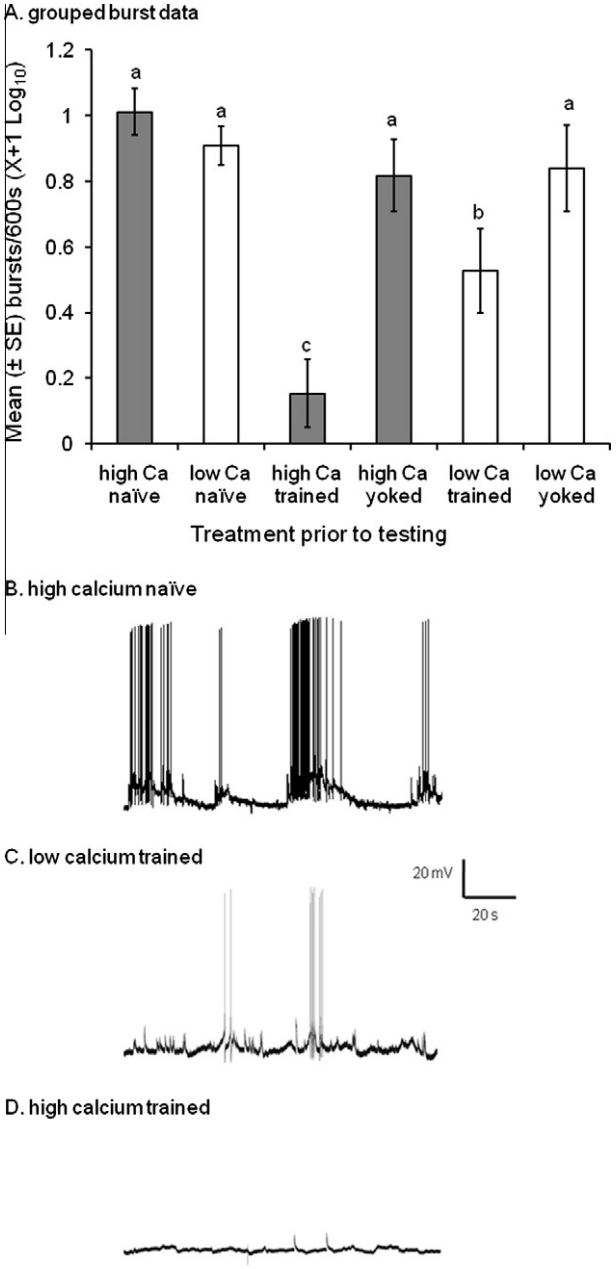

Fig. 3. The number of action potential bursts fired from RPeD1 in 600 s. (A) Comparison of mean bursting behavior between untrained (naïve), trained and yoked snails maintained for 1 week in either pond water containing high (80 mg/l) calcium (shaded bars) or pond water containing low (20 mg/l) calcium (white bars). Recordings from trained and yoked snails occurred 24 h after training. Different lower case lettering = significantly different (SNK: $P < 0.05$). Representative RPeD1 electrophysiological traces of action potential bursts recorded from: (B) a naïve snail maintained in pond water containing high calcium, four bursts of action potentials are seen, (C) a trained snail maintained in low calcium pond water, two bursts of action potentials are seen and (D) a trained snail maintained in high calcium pond water, no bursts are seen. The traces in (C) and (D) were recorded 24 h after training.

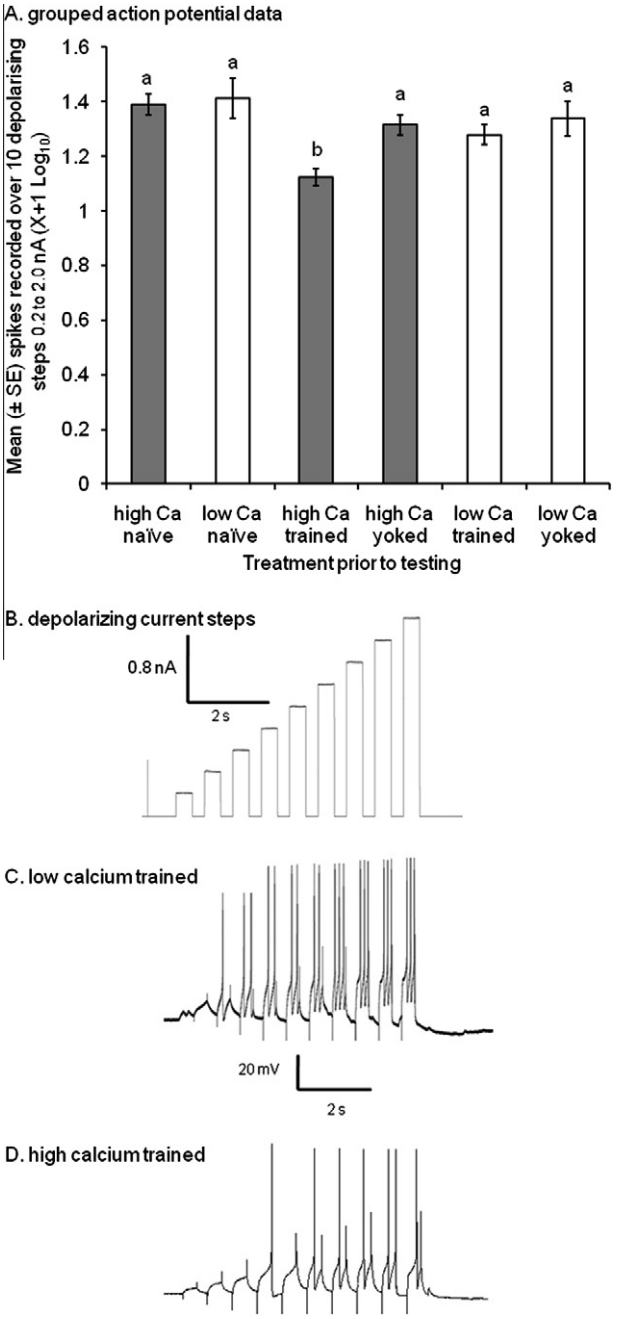

Fig. 4. Excitability of RPeD1 as measured by the number of action potentials fired in response to depolarizing current steps over 10 s (see Fig. 3). (A) Comparison between mean response in untrained (naïve), trained and yoked snails maintained for 1 week in pond water containing high (80 mg/l) calcium (shaded bars) or pond water containing low (20 mg/l) calcium (white bars). Recordings from trained and yoked snails occurred 24 h after training. Different lower case lettering = significantly different (SNK: $P < 0.05$). Representative electrophysiological traces demonstrating the protocol for measuring RPeD1 excitability: (B) the depolarizing current steps that the cells were exposed to, (C) voltage responses recorded from the RPeD1 neuron of a low (20 mg/l) calcium snail 24 h after training and (D) voltage responses recorded from the RPeD1 neuron of a high (80 mg/l) calcium snail 24 h after training.

preparations recorded 24 h after 'training', irrespective of whether the snails were maintained in high or low environmental conditions (SNK: $P > 0.05$). The second point is that while the 1TT in snails exposed to low environmental calcium did not result in LTM, such training did significantly alter the electrophysiological properties of RPeD1. That is, the number of bursts recorded in RPeD1 of these trained snails that did not exhibit LTM was significantly less than the number of bursts in the naïve or yoked low calcium control snails (SNK: $P < 0.05$ for all significant differences).

The final and most important point is that in snails that received the 1TT procedure in high calcium and exhibited LTM behaviorally had significantly fewer bursts than those preparations trained in low calcium and the control preparations that did not demonstrate a reduction in the number of pneumostome opening attempts (SNK: $P < 0.05$ for all pair-wise comparisons). That is, in snails where behavioral memory was found, an electrophysiological

correlate was also present, i.e., there were significantly fewer bursts than in either yoked snails, untrained snails or snails trained in low Ca that show no behavioral memory.

The decrease in the bursting of RPeD1 following 1TT, especially from the recordings of RPeD1 in the 1TT high calcium preparations could be related to an overall decrease in excitability of the neurons. To test this possibility, we subjected the RPeD1 neurons to a series of depolarizing steps to determine if there was a significant difference in the number of action potentials evoked in RPeD1 neurons from the low calcium trained snails compared to the number elicited in RPeD1 neurons from the high calcium trained snails. Group data are presented in Fig. 4A; representative data are shown in Fig. 4B–D. As can be seen, when subjected to depolarizing current steps (Fig. 4B), there was a difference in the number of action potentials fired by the RPeD1 neuron from the low calcium trained snail (Fig. 4C) and the high calcium trained snail (Fig. 4D). When grouped data were compared (Fig. 4A), it can be seen that there were no significant differences in the number of action potentials elicited in the RPeD1 neurons in the naïve high, naïve low, high calcium yoked, trained in low calcium and yoked low calcium; however, there was a significant decrease in the number of action potentials evoked in the RPeD1 neurons from preparations that received 1TT in high calcium compared to all the other conditions (Fig. 4A; one-way ANOVA: $F_{5,55} = 4.31$, $P = 0.002$; SNK $P < 0.05$ for all pair-wise comparisons between snails trained in high environmental calcium and other groups). That is, in snails trained in high calcium that demonstrated LTM, RPeD1 was less excitable compared to neurons from preparations of naïve, yoked and trained in low calcium individuals, as measured by the number of action potentials elicited by the depolarizing steps.

It is possible that the decreased excitability of RPeD1 neurons from the 1TT high calcium preparations was due to a significantly lower membrane input resistance. To test this possibility, we measured the input resistance of RPeD1 in naïve, yoked control and 1TT high and low calcium preparations (Fig. 5). When a series of hyperpolarizing current pulses are passed through RPeD1, the slope of the resulting voltage changes provides a measure of input resistance. Grouped data are presented in Fig. 5A and representative data are shown in Fig. 5B and C. According to Ohm's Law ($V = IR$), a lower input resistance should result in a neuron that is less likely to fire an action potential for a given depolarizing current. The representative data (Fig. 5B and C) show that the RPeD1 neuron from a 1TT low calcium preparation had a larger input resistance that an RPeD1 neuron from a 1TT high calcium preparation. When we examined the grouped data we found the following: (1) there was no significant difference in the input resistance in RPeD1 neurons from naïve vs. yoked control (both high and low calcium) preparations (SNK: $P > 0.05$); (2) the input resistance of RPeD1 neurons from naïve low calcium snails was significantly lower than from naïve high calcium snails (SNK: $P < 0.05$), but did not differ from either yoked control group (SNK: $P > 0.05$); (3) input resistance of RPeD1 neurons from 1TT low calcium did not differ significantly from naïve low calcium or low calcium yoked preparations (SNK: $P > 0.05$) and (4) the input resistance of RPeD1 neurons from 1TT high calcium preparations was significantly lower than that from any other group (Fig. 5A; one-way ANOVA: $F_{5,55} = 7.38$, $P < 0.001$; SNK: $P < 0.05$ for all pair-wise comparisons). That is, RPeD1 neurons from preparations that exhibit LTM (i.e. snails receiving the 1TT in high environmental calcium) had a significantly lower membrane input resistance than RPeD1 neurons from any of the yoked control, naïve or 1TT low environmental calcium groups. Thus, we conclude that there are significant differences in intrinsic membrane properties between RPeD1 neurons from preparations exhibiting LTM compared to preparations (naïve, yoked controls, and 1TT low environmental calcium) that do not exhibit LTM.

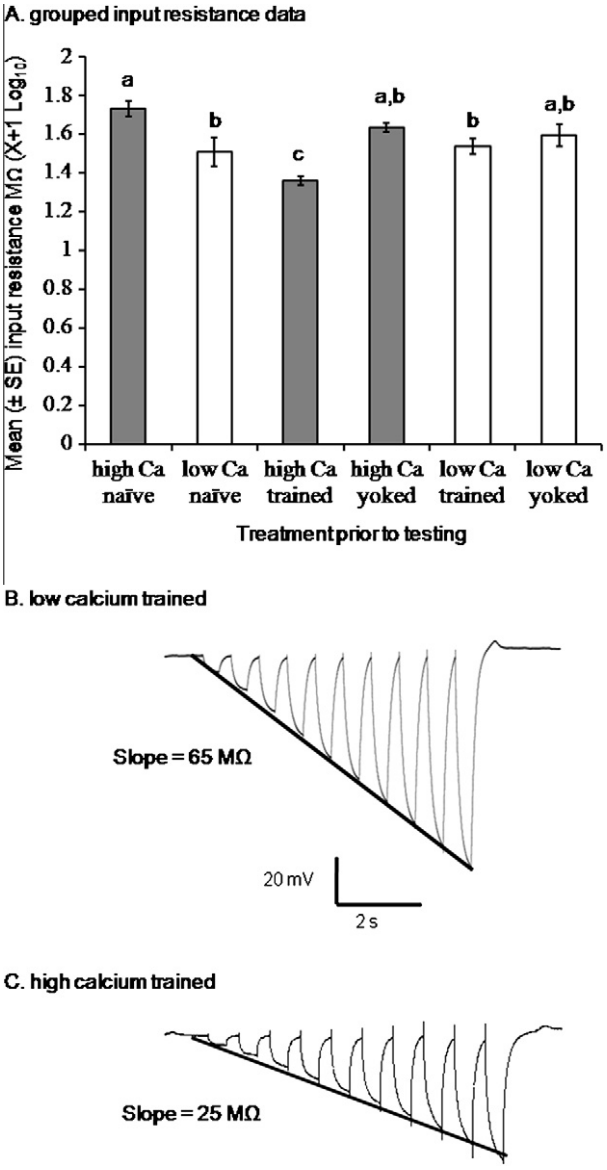

Fig. 5. Input resistance of RPeD1 calculated from hyperpolarizing current steps (RPeD1 was exposed to increasingly hyperpolarized current steps and the resulting voltages were recorded). (A) Comparison between the mean response from untrained (naïve), trained and yoked snails maintained in pond water containing high (80 mg/l) calcium (shaded bars) or pond water containing low (20 mg/l) calcium (white bars). Recordings from trained and yoked snails occurred 24 h after training. Different lower case lettering = significantly different (SNK: $P < 0.05$). Representative electrophysiological recordings demonstrating the protocol for measuring input resistance: (B) Voltage trace recorded from a trained snail maintained in low calcium pond water and (C) a trained snail maintained in high calcium pond water. The traces in (A) and (B) were recorded 24 h after training. The steeper the slope of the voltage trace, the larger the resulting resistance.

4. Discussion

L. stagnalis is frequently used as a model organism to study behavior and physiology due to its relatively simple neuronal network, predictable set of behaviors, the tractability of those behaviors and the ease with which they can be reared in the laboratory (see reviews in: Benjamin, Staras, & Kemenes, 2000; Brembs, 2003; Lukowiak et al., 2003, 2010; Parvez, Rosenegger, Orr, Martens, & Lukowiak, 2006). Whilst it is well known that *L. stagnalis* is a calciphile, and requires environmental calcium $\geqslant$20 mg/l for growth

and survival (Boycott, 1936; Van Der Borght & Van Puymbroek, 1966; Young, 1975a), there is very little information on the effect of environmental calcium, within ecologically relevant concentrations, on its behavior and physiology. In addition, there is considerable evidence that environmental calcium is declining in freshwater systems in North America and Europe. For example, a 35% decline in environmental calcium available in some freshwater systems has occurred over the past two decades (Jeziorski et al., 2008; Keller, 2009). We have previously demonstrated in *L. stagnalis* that acute exposure to a low calcium environment alters basic physiological and behavioral traits, as exemplified by alterations in respiration and locomotion (Dalesman & Lukowiak, 2010). Here we demonstrated that a 1 h to 1 week acute exposure to a low calcium environment blocked LTM formation and reduced the neural correlates of LTM in a neuron, RPeD1, which is necessary for LTM formation. These results have important implications for the study of adaptive behavior and physiology in freshwater gastropods.

In *L. stagnalis*, LTM formation, as a result of operant conditioning of aerial respiration, can be modified (enhanced or suppressed) by stress (Lukowiak et al., 2008, 2010). LTM can be enhanced following exposure to sympatric predator cues (Orr & Lukowiak, 2008; Orr et al., 2009) or blocked by cooling (Sangha et al., 2003) and crowding (De Caigny & Lukowiak, 2008), all of which are environmentally relevant stressors. In addition to requiring environmental calcium for growth and development, a low calcium environment both increases the basal metabolic demand of *L. stagnalis* and reduces the energy available for behaviors such as locomotion (Dalesman & Lukowiak, 2010). Hence, we hypothesized that a low calcium environment, at a concentration experienced by field populations, would act as a stressor for this species. However, until we performed the experiments shown here, it was unclear to us whether this stressor would enhance or block LTM formation. We found that *L. stagnalis* were capable of forming intermediate-term memory (ITM) in low environmental calcium, hence this stressor did not prevent the learning process nor the formation of a memory that is only dependent on the translation of pre-existing mRNA (i.e. ITM: Sangha et al., 2003). However, *L. stagnalis* were unable, as a result of their exposure to the low calcium environment either for 1 week or 1 h, to form long-term memory (LTM). Therefore, we propose that the low calcium environment may be preventing the required transcription processes in neurons (e.g. RPeD1) necessary for LTM formation. Moreover, the suppressive effect of low environmental calcium on LTM formation occurred rapidly, and likely depends on sensory structures such as the osphradium to initiate alterations in genomic processes necessary to protect calcium stores. The fact that memory formation was simultaneously inhibited suggests that these genomic changes were incompatible with LTM formation. These data contrast with data from snails held at the high environmental calcium for as little as 1 h, which were capable of forming both ITM and LTM. Thus, the detection of sufficient environmental calcium (i.e., 80 mg/l) rapidly reversed the genomic changes in neurons (e.g. RPeD1) induced by the low environmental calcium, such that the necessary molecular events that cause the formation of LTM can ensue. Moreover, in the high calcium concentration this memory did not extend to 48 h, concurring with previous findings following one-trial conditioning (Martens et al., 2007).

RPeD1 is part of a three neuron network, a central pattern generator (CPG) that is both necessary and sufficient to drive aerial respiratory behavior in *L. stagnalis* (Syed et al., 1990, 1992). Moreover, since we were examining a form of non-declarative memory, there is a high probability that the site of memory formation and storage is in the same neuronal network that mediates the behavior (Milner, Squire, & Kandel, 1998). Thus, it was not too surprising to find that the ablation of RPeD1's soma, which removes the nucleus but leaves intact its primary neurite, results in a snail that can learn and form ITM, but cannot form LTM, undergo reconsolidation or extinction (Sangha, Scheibenstock, & Lukowiak, 2003; Sangha et al., 2003; Scheibenstock et al., 2002). Changes in some electrophysiological properties of RPeD1 have been found following an alternate associative learning training procedure, which results in LTM. In that procedure the pneumostome was physically 'poked' as it attempts to open, and the training results in a significant reduction in aerial respiration in both semi-intact preparations made from previously trained snails, or from naïve preparations trained 'in the dish' (McComb et al., 2005; Spencer, Syed, & Lukowiak, 1999). Additionally, these electrophysiological changes were correlated with molecular changes necessary for LTM formation that occur in RPeD1 and other CNS neurons (Rosenegger, Wright, & Lukowiak, 2010). Here we tested whether similar electrophysiological changes also occur in RPeD1 following the 1TT procedure in alternate external calcium environments, both conducive and detrimental to LTM formation. We found that the 1TT procedure in the high calcium environment (80 mg/l) resulted in LTM formation. Further, we found that the electrophysiological properties of RPeD1 in these snails were similar to those found using the more traditional training procedure (i.e. a 'poke' to the pneumostome as it attempts to open). That is, whether assessed by the number of spontaneously occurring bursts of action potentials, or the number of evoked action potentials with discreet depolarizing current steps, there was a significant decrease in the excitability of RPeD1. The concomitant decrease in the input resistance suggests that the decrease in the excitability of RPeD1 may have been due to an increase in basal ionic flux across the membrane. As resistance fell, the current required to initiate spike firing became proportionally higher, and it became more difficult to initiate the burst of action potentials that result in pneumostome opening. This can be seen when comparing the depolarizing currents required to initiate action potential firing in RPeD1. In naïve, high calcium snails, 0.2 nA of depolarizing current was sufficient to induce firing in 92% of the snail preps, with 0.4 nA resulting in 100% of the preparations firing action potentials. Conversely, following training, preparations made from high calcium snails fired no action potentials at 0.2 nA of depolarizing current and only 11% of the preps fired at 0.4 nA. It took a 1.2 nA depolarizing current to induce firing in all of the trained, high calcium preparations. When snails were trained and tested in low environmental calcium, this significant decrease in electrophysiological activity relative to naïve snails was reduced. Confirmation that the reduction in activity was due to the training procedure was demonstrated by the absence of a significant difference in activity between the naïve snails and yoked controls that had not experienced the KCl bath contingent with pneumostome opening.

When *L. stagnalis* were trained in low environmental calcium, there was still a significant reduction in the number of action potential bursts fired compared to both yoked controls and naïve, snails. This indicates that despite no behavioral changes, the 1TT procedure had altered the electrophysiological properties of RPeD1 to a degree, but not to the extent needed to see a behavioral change. These data are reminiscent of findings from the 'poking' training procedure used in our laboratory to reduce aerial respiration, where it was discovered that following a training session that was insufficient to create LTM, if the snails received an additional training session (normally producing ITM) on the following day, they would now possess LTM (Parvez, Stewart, Sangha, & Lukowiak, 2005). This suggests that even 24 h following the ITM training protocol, snails, while no longer showing behavioral memory, still possess a residual memory trace which allowed a single additional training session, that normally only results in ITM, to now produce LTM (Parvez et al., 2005). While the previous study was based on behavioral data alone, the electrophysiological data obtained in the present study provide strong support for the residual memory trace

hypothesis, that 24 h following training in snails without any behavioral traces of memory, RPeD1 remained in a significantly different state from both the naïve and yoked control snails.

The electrophysiological data obtained here support previous findings concerning neural correlates of LTM following operant conditioning of aerial respiratory behavior in *L. stagnalis*. Spencer et al. (1999) reported no significant differences in the RMP of RPeD1 following operant conditioning. In that report, however, the authors recorded from RPeD1 in isolated ganglionic preparations. That is, the CNS was removed from the snails prior to recording from RPeD1. We now know that the periphery (i.e., pneumostome and osphradium area and osphradial ganglion) contributes both excitatory and inhibitory inputs to neurons in the CNS (Il-Han, Janes, & Lukowiak, 2010; McComb, Meems, Syed, & Lukowiak, 2003). Thus, data obtained from only ganglionic preparations may not be an accurate reflection of the 'state of the nervous system' following conditioning. Subsequently, Spencer et al. (2002) recorded from RPeD1 in a semi-intact preparation 1 h after training, and also reported no change in the RMP of RPeD1 in preparations made from snails that had exhibited 'good' memory. They did find, that in these semi-intact preparations, there was a significant decrease in spontaneous activity in RPeD1 following a presentation of a tactile stimulus to the pneumostome area. This decrease in activity did not occur in control preparations. Naïve snail preparations trained in vitro showed no change in the RMP of RPeD1 following in vitro training (McComb et al., 2005). Recent work demonstrated that in adult and juvenile snails, trained with the 1TT procedure in normal pond water (i.e., 80 mg/l environmental calcium), there was also no significant change in the RMP of RPeD1 (Sunada, Horikoshi, Lukowiak, & Sakakibara, 2010). Of interest, these authors did find that in both juvenile and adult snails trained with the 1TT procedure in the presence of the scent of a predator (i.e., crayfish kairomones in 80 mg/l environmental calcium), that the RMP of RPeD1 was significantly more hyperpolarized compared to control preparations. It appears that the significant change in the RMP of RPeD1 may primarily be due to the detection of the predator, as Orr et al. (2007) demonstrated that predator detection results in a hyperpolarization of RPeD1. This appears to be mediated by a serotonergic system, stimulated by predator detection via the osphradium (Il-Han et al., 2010).

Previous studies in mollusk and other model system preparations have found that there were alterations in synaptic connectivity that were correlated with the presence of behavioral LTM (Kandel, 2001). However, other data in both mollusk and mammalian model systems, show that behavioral LTM is correlated not only with plastic changes in synaptic connectivity, but also with changes in the intrinsic membrane properties of neurons, similar to that we observed here (Alkon et al., 1985). These changes in intrinsic membrane properties, that alter the excitability of the neuron, have been referred to as 'non-synaptic substrates' of LTM (Nikitin et al., 2008). These changes may involve alterations in channel activity that leads to either increased or decreased neuronal excitability, and hence membrane input resistance. For example, changes in calcium, potassium or sodium channel activity have been hypothesized to account for an increased neuronal excitability in various vertebrate and mammalian model systems, that correlates well with behavioral LTM (Alkon & Sakakibara, 1985; Alkon et al., 1985; Frost, 2006; Magee & Johnston, 2005; Marder et al., 1996; Nikitin et al., 2006). Here we showed, that following the 1TT procedure in high environmental calcium (which leads to behavioral LTM), there are significant alterations in neuronal excitability seen, both with spontaneously occurring inputs, and when the neuron is driven by depolarizing or hyperpolarizing current injections. Thus, our findings here add to the growing body of evidence that a change in membrane excitability contribute to how the nervous system encodes LTM.

The disruptive effect of low environmental calcium on the formation of LTM was rapidly reversed by just 1 h of exposure to high environmental calcium. That was, snails held in the low calcium environment for 1 week only require 1 h exposure to a high calcium environment to regain their ability to form LTM. The reverse is also seen. The rapid reversal in the ability to form LTM after training snails in an alternate calcium environment indicates that the mechanism by which the sensing of calcium alters memory is not related to haemolymph calcium concentration. *L. stagnalis* is able to maintain calcium concentration in the haemolymph for over a week, even in the complete absence of environmental calcium (Dewith, 1977). This species is also able to orientate towards a high calcium environment in choice tests, indicating that they may be able to directly sense environmental calcium concentration (Piggott & Dussart, 1995). The rapid behavioral and electrophysiological response to the external environmental calcium concentration provides further evidence that this may be the case.

Whilst snails could not form LTM in low environmental calcium, low environmental calcium did not prevent the recall of a previously established memory that occurred when the snails were trained in the high environmental calcium. These data further argue against the possibility that exposure to low environmental calcium alters the internal haemolymph calcium. If the haemolymph calcium was altered by the acute exposure to the low environmental calcium, then we would have expected that an already established memory should be as affected by the low environmental calcium, as is the establishment of a new memory. Our working hypothesis is that snails detect low environmental calcium, possibly by sensory neurons located in the osphradium, and that the activity of these neurons alter the molecular machinery in RPeD1, such that the molecular steps necessary for the memory consolidation to produce behavioral LTM cannot occur. If, however, memory consolidation has already occurred, as the result of changes in neuronal excitability and synaptic connectivity, the molecular changes in the neurons, such as RPeD1, induced by the detection of the low environmental calcium are not sufficient to reverse the changes in neuronal excitability and synaptic connectivity.

Calcium fluctuations in the freshwater habitat are common (Macan, 1950; McKillop & Harrison, 1972; Williams, 1970; Dalesman unpublished data), such that freshwater gastropods may experience ten-fold changes in calcium concentration over the course of a year. Whilst *L. stagnalis* populations are normally found in environments of $\geqslant$20 mg/l calcium, they may experience periods below or above this level. The data we present here indicate that whilst experiencing low environmental calcium *L. stagnalis* will be less able to demonstrate behavioral plasticity. One consequence of reduced memory duration in low environmental calcium, for example, may lead to a lack of adaptability to the predation environment. *L. stagnalis* has enhanced innate responses to predators (Dalesman, Rundle, & Cotton, 2007; Orr et al., 2007) and also heterospecific alarm cues from species that overlap in distribution (Dalesman, Rundle, Bilton, & Cotton, 2007); however recognition of predatorial threat can also be improved through learning, both in terms of recognizing the predator (Dalesman, Rundle, Coleman, & Cotton, 2006; Dalesman, Rundle, & Cotton, 2009) and also heterospecific alarm cues (Dalesman & Rundle, 2010). The data presented here suggest that the ability to remember stressful events, such as those that might be associated with a predator encounter, may be impeded in low environmental calcium environments. These data indicate that *L. stagnalis* found naturally in low calcium environments, or during periods of low calcium availability such as following a flooding event, may be less able to adapt to novel stressors than populations found in harder (i.e., high calcium) waters. This, together with an inability to express induced morphological defenses (e.g. specific shell characteristics; Rundle et al., 2004), may reduce fitness of *L. stagnalis* to a

greater extent than would be predicted by lower growth and reproductive output in low calcium environments alone. The strong effect that environmental calcium concentration has, on physiology and behavior in response to training to reduce aerial respiration in *L. stagnalis*, suggests that it is also an element of gastropod husbandry that needs to be carefully considered when studying other traits.

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