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Review

The perception of stress alters adaptive behaviours in *Lymnaea stagnalis*

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Summary

Stress can alter adaptive behaviours, and as well either enhance or diminish learning, memory formation and/or memory recall. We show here that two different stressors have the ability to alter such behaviours in our model system, *Lymnaea stagnalis*. One, a naturally occurring stressor – the scent of a predator (crayfish) – and the other an artificially controlled one – 25 mmol l^{-1} KCl – significantly alter adaptive behaviours. Both the KCl stressor and predator detection enhance long-term memory (LTM) formation; additionally predator detection alters vigilance behaviours. The predator-induced changes in behaviour are also accompanied by specific and significant alterations in the electrophysiological properties of RPeD1 – a key neuron in mediating both vigilance behaviours and memory formation. Naive lab-bred snails exposed to crayfish effluent (CE; i.e. the scent of the predator) prior to recording from RPeD1 demonstrated both a significantly reduced spontaneous firing rate and fewer bouts of bursting activity compared with non-exposed snails. Importantly, in the CE experiments we used laboratory-reared snails that have not been exposed to a naturally occurring predator for over 250 generations. These data open a new avenue of research, which may allow a direct investigation from the behavioral to the neuronal level as to how relevant stressful stimuli alter adaptive behaviours, including memory formation and recall.

Key words: *Lymnaea*, instinct, aerial respiration, long-term memory, crayfish predator, vigilance behaviours.

Introduction

The perception of stress by sensory systems modulates adaptive behaviours including memory formation and/or its recall. This has been noted in the scientific literature since the time of Bacon (Bacon, 1620) but it is probably best summarized in the so-called Yerkes–Dotson 'law' (Yerkes and Dotson, 1908; Shors, 2004). This 'law' is depicted graphically in Fig. 1. As can be seen stress and/or attention is an important element in determining both whether and how 'good' information becomes stored as long-term memory (LTM). Since every organism internalizes and retains details of its surroundings to increase its chance of survival, it is not surprising that memory is demonstrated in all animals. However, it is impractical to encode all events into memory. Thus, organisms should only expend the 'neuronal cost' (e.g. altered gene activity and new protein synthesis) necessary to form LTM to 'relevant' events. One very important factor that helps determine whether a specific occurrence will be encoded into memory is the level of stress of the organism at the time of the event. Because memory is dynamic, stress and traumatic events have substantial modulatory effects on memory, including false memory and post-traumatic stress syndrome (Kim and Diamond, 2002; Lukowiak et al., 2007; Yehuda and LeDoux, 2007). These effects have been studied in a number of different model organisms, with sometimes contradictory results (Shors, 2004). That is, in some instances memory is enhanced whilst in others its formation or its recall is blocked. Given the complexities of the vertebrate brain and animal behaviour, and the diverse ways stressors act on memory formation, disagreement in the literature is not surprising.

The vast majority of the learning and memory studies involving *Lymnaea* have utilized laboratory-bred specimens of *Lymnaea* (for reviews, see Benjamin et al., 2000; Lukowiak et al., 2003b; Brembs, 2003; Parvez et al., 2006b) and the majority of these snails have been derived from snails originally collected (in the 1950s) from canals in a polder in Utrecht Provence in the Netherlands. Subsequently these were maintained at Vrije Universeit in Amsterdam and then distributed to various labs throughout the world. However, it is important to remember that in the wild, *Lymnaea* respond to a variety of predator-released kairomones (a chemical secreted and released by an organism that, when detected by an organism of another species, evokes a response that adaptively favours the latter) and, depending on the specific predator detected, utilize a functionally appropriate response (e.g. sheltering under crevices for fish or crawling above the waterline for crayfish (Dalesman et al., 2006; Jacobsen and Stabell, 2004; Covich et al., 1994; Rundle and Bronmark, 2001). Thus, snails follow the age-old axiom that the most effective way to avoid becoming prey is to avoid the predator. Many examples of species-typical behaviours labelled 'vigilance' or 'risk assessment' following predator detection have been demonstrated (Apfelbach et al., 2005). Prey species assess the risk of predation and adjust their behavioural and/or anatomical phenotype appropriately (Hoverman and Relyea, 2007; Kats and Dill, 1998; Turner et al., 2000; Orr et al., 2007). Here we tested whether lab-reared *Lymnaea* have maintained the ability to both detect and respond appropriately (i.e. make the correct decision) to the scent of a crayfish predator, even though they have not experienced a crayfish predator for over 250 generations. Since the vast majority of previous reports (e.g. Chivers et al., 1996; Hazlett et al., 2002; Langerhans and Dewitt, 2002; Rochette et al., 1998) concerning how gastropods (including *Lymnaea*) respond to predator detection (*via* kairomones interacting with sensory neurons) utilized

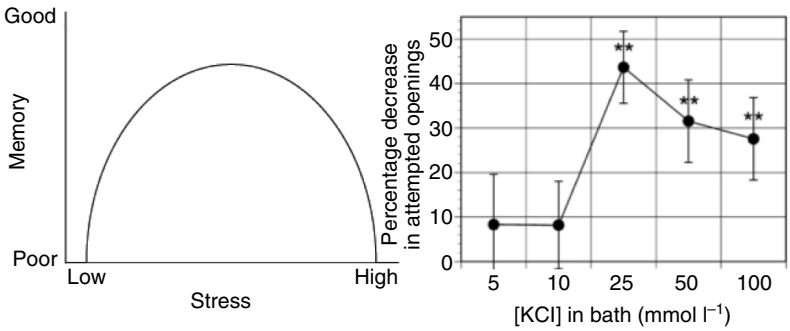

Fig. 1. The 'Yerkes-Dodson law' is derived from experiments performed in the early 1900s and as plotted here (left) demonstrates a relationship between stress or arousal and performance. That is, in this conceptual scheme memory formation gets better with increasing stress, but only to a certain point: when levels of stress become too high, the ability to form memory decreases. A similar curve (right) has been experimentally derived with increasing concentrations of KCl as a stressor in *Lymnaea* [reprinted from Martens et al. (Martens et al., 2007a), with permission from Elsevier]. Briefly, when a 5 mmol l^{-1} concentration of KCl was used in the bath, memory was not observed. With concentrations of KCl greater than 25 mmol l^{-1} memory was obtained, but the optimal concentration of KCl was 25 mmol l^{-1} as with increasing concentrations the resultant memory was not as robust.

wild-caught animals it makes it difficult to determine whether predator-induced defence responses were innate or the result of prior experience (Dalesman et al., 2006). However, because we were using lab-bred snails we were able to surmount this problem.

In this review we will primarily concentrate on one specific behaviour in *Lymnaea* when we look at memory formation and stress; aerial respiratory behaviour. *Lymnaea* is a bimodal breather that satisfies its respiratory needs either cutaneously, through the skin, or aerially through the pneumostome, the respiratory orifice (Lukowiak et al., 1996). Aerial respiration, which involves opening the pneumostome at the water's surface to allow atmospheric gas exchange, is driven by a three interneuron central pattern generator (CPG) whose sufficiency and necessity has been directly demonstrated (Syed et al., 1990; Syed et al., 1992). In hypoxic conditions the frequency of aerial respiration increases and can be modified by operant conditioning (Lukowiak et al., 1996; Lukowiak et al., 1998; Lukowiak et al., 2003a; Lukowiak et al., 2003b; Parvez et al., 2006b). Briefly, a tactile stimulus is applied to the pneumostome area each time the snail begins to open it for gas exchange. Depending on the training procedure used, intermediate-term memory (ITM; persists for up to 3 h and depends on *de novo* protein synthesis) or LTM (persists for more than 6 h and depends on both altered gene activity and *de novo* protein synthesis) can be formed (Lukowiak et al., 2000; Scheibenstock et al., 2002; Sangha et al., 2003a; Sangha et al., 2003b). In fact, molecular changes in one of the three CPG neurons, RPeD1, have been shown to be absolutely necessary for LTM formation, extinction, memory reconsolidation and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003c; Sangha et al., 2003d; Sangha et al., 2005; McComb et al., 2005a; Lattal et al., 2006). We will thus have the opportunity to determine how stress in *Lymnaea* alters LTM formation and vigilance behaviours at the single neuron level.

To evoke a standardized, repeatable, acute stress response in *Lymnaea*, the snails were exposed to a noxious stimulus, 25 mmol l^{-1} KCl [i.e. the KCl bath; see Martens et al. (Martens et al., 2007a; Martens et al., 2007b) for full details]. The other stressor that will be discussed is exposure to the 'smell' of a crayfish predator. To obtain this 'smell', which we call crayfish effluent (CE), the crayfish were maintained in aquaria and we used the water taken from the aquarium to train the snails in. Thus, snails did not come into direct contact with the predator but only came into contact with water taken from the aquarium where the crayfish were maintained (i.e. the CE).

KCl as a stressor

A single 30 min training session results in a memory that persists for approximately 3 h (i.e. ITM) but not for 24 h (Lukowiak et al., 1998; Parvez et al., 2005; Parvez et al., 2006a; Parvez et al., 2006b). However, we found (Martens et al., 2007a) that if we stressed the snails with a KCl bath either immediately before or just after (see Martens et al., 2007a) the 30 min training session, LTM resulted (Fig. 2). That is, memory now persisted for at least 24 h. Thus, it appears that the perception of a noxious, stressful stimulus (i.e. the KCl bath) that elicits the whole-body withdrawal response (Inoue et al., 1996) is sufficient to cause a training procedure (i.e. a single 30·min training session) that normally does not result in LTM formation to now result in LTM. However, a number of controls had to be performed before we could conclude that the KCl stressor enhanced LTM formation.

We first needed to control for the handling of the snails (i.e. picking them up from the beaker and placing them in the Petri dish). That is, handling might be a sufficiently stressful event (as it sometimes is in

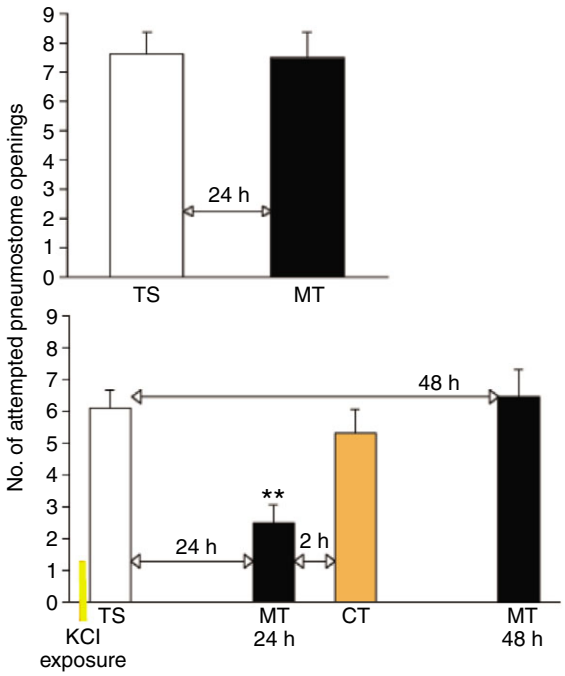

Fig. 2. A KCl stressor enhances LTM formation in *Lymnaea*. Top, snails (N=20) that received one 30 min training session (TS) of contingent 'poking' did not have a significant change in breathing behaviour when tested 24 h later (MT; top). Bottom, snails (N=38) that were exposed to 25 mmol l^{-1} KCl for 30–35 s before a 30 min TS exhibited memory at 24 h (**P<0.01). When tested in carrot context (CT) the number of attempted pneumostome openings returned to naive levels (P>0.05), indicating context-specific memory. Snails that received KCl before training and were tested for savings at 48 h did not show memory. [Reprinted from Martens et al. (Martens et al., 2007a), with permission from Elsevier.]

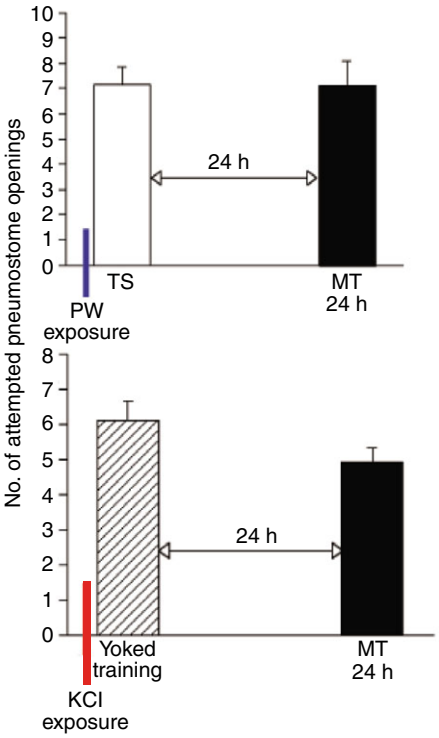

Fig. 3. Controls for the KCl bath experiments. Top, snails (*N*=19) that were placed in pond water (PW) rather than the KCl bath before 30 min of training did not have memory at 24 h. Bottom, snails (*N*=12) that were exposed to KCl and then yoke (i.e. non-contingently) trained showed no significant difference in the number of attempted pneumostome openings from naive levels, demonstrating that LTM was not formed. Reprinted from *Neurobiology of Learning and Memory* **87**, 391–403 (2007) with permission from Elsevier (Martens et al., 2007a).

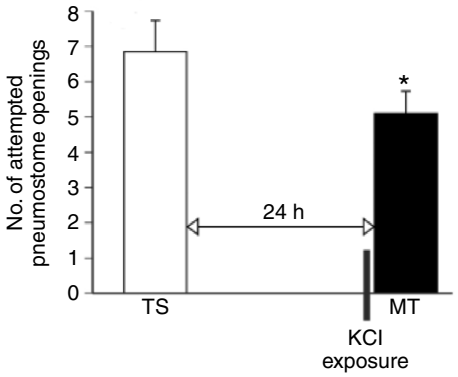

Fig. 4. A stressful event, the KCl bath, can improve memory at the time of recall. Snails (*N*=20) that were trained for 30 min and then only received KCl exposure before the memory test (MT) 24 h later exhibited memory when tested (**P*<0.05). [Reprinted from Martens et al. (Martens et al., 2007a), with permission from Elsevier.]

rodents) to enhance LTM formation. Thus, a cohort of snails (Fig. 3) were placed in the same Petri dishes for 30–35 s both immediately before and after training; however, instead of the KCl bath the dishes contained only PW from their home aquarium. When tested 24 h later these snails also did not exhibit LTM; that is, there was no difference in the number of attempted pneumostome openings in the MT compared with the TS. Thus, handling of snails was not a sufficiently stressful event to enhance LTM formation. It needs to be pointed out that this handling of snails results in the whole-body withdrawal response. That is, when picked up and placed into the Petri dish the snail typically withdraws into its shell. These data also demonstrate that activation of the whole-body withdrawal response is not in itself sufficient to cause LTM enhancement. While handling snails in the manner described (i.e. placing them in the Petri dish filled with PW) does result in the snail withdrawing into its shell, these snails begin to explore their new environment significantly faster than those placed in the KCl bath. Typically, snails in the KCl bath never came out of their shell as long as they were in the bath. We interpret this to indicate that the combination of the KCl and handling is a 'stronger' stressor than handling and PW.

We also found that the perception of the stressful stimulus itself did not lead to LTM formation (Fig. 3, bottom). That is, placing snails in the KCl bath just prior to a yoked control training session (non-contingent application of the stimuli) did not result in LTM formation.

The perception of a stressful situation immediately before an attempt to recall memory can be detrimental to recall (see Shors, 2004). To determine whether the stressor used above (i.e. the KCl bath) would be detrimental to memory recall, snails were trained with a protocol previously shown to result in LTM (Lukowiak et al., 1998; Taylor and Lukowiak, 2000; Sangha et al., 2003a); two 30 min tactile training sessions, between which the snails were returned to the home aquarium for 1 h. Twenty-four hours later these snails were given the KCl bath, then placed in the hypoxic training beaker and tested for LTM. We found that LTM was present (data not shown) (see Martens et al., 2007a). Thus, in *Lymnaea* this stressor did not block memory recall.

We next found a very surprising result (Fig. 4). We trained a cohort of naive snails with a single 30 min tactile training session that was neither preceded nor followed by a KCl bath. The next day these snails were placed in a 25 mmol l^{-1} KCl bath immediately before a MT. These snails showed LTM despite the initial training protocol being insufficient to establish LTM. Therefore, we hypothesize that the application of the KCl bath just before the MT reinstated or potentiated a residual memory trace that was sufficient to cause the formation of LTM [i.e. altered gene activity and new protein synthesis (Parvez et al., 2005)]. Our lab has previously demonstrated that ITM training, which leaves no evidence of memory at 24 h, results in a 'memory substrate' that an additional ITM training session can build upon, resulting in LTM 24 h later (Parvez et al., 2005; Parvez et al., 2006a; Parvez et al., 2006b). Here, it appears that the application of a KCl bath before the MT salvages the 'residual memory substrate' to allow LTM formation.

We had previously demonstrated (Scheibenstock et al., 2002) that the soma (i.e. where the genes are) of RPeD1 had to be present for LTM formation when a training procedure (two 30 min training sessions separated by a 1 h interval) was used. We wished to determine whether the same requirement for RPeD1's soma was necessary for the memory enhancement brought about by the KCl stressor. These data are shown in Fig. 5. As can be seen in those snails where RPeD1's soma had been ablated 2 days previously, the KCl stressor did not cause LTM formation. In the control groups ablation of another neuron's soma, VD1, which is approximately the same size as RPeD1 but which is not involved in driving aerial respiratory behaviour, did not block KCl's ability to enhance LTM formation nor did the sham-operated control. Thus, for the KCl stressor to enhance LTM formation the soma of RPeD1 must be intact.

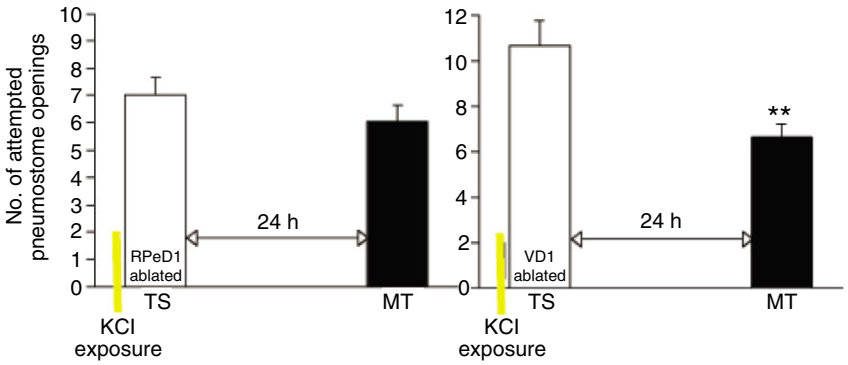

Fig. 5. The soma of RPeD1 is required for memory formation with the KCl bath training procedure. Snails (N=22) that had the soma of RPeD1 ablated 2 days before training were placed in a 25 mmol l^{-1} KCl bath and then trained for 30 min (TS). A day later (MT) the number of attempted pneumostome was statistically the same as in TS. Thus LTM was not formed. On the other hand, snails (N=14) that had the soma of VD1 ablated 2 days prior to training did have memory at 24 h (**P<0.01).

Is too much stress a bad thing, as regards memory enhancement, as predicted by the Yerkes–Dodson 1908 'law'? We tested this question in two ways. The first way was to increase the concentration of the KCl bath; but when we used 100 mmol l^{-1} KCl the snails just got unresponsive (i.e. sick). So, instead, we subjected snails to the KCl bath immediately before and immediately after the 30 min training session. A single application of this stressor either before or after the single 0.5 h training session enhanced LTM formation; however, when applied both before and after training, LTM formation was blocked (Fig. 6) showing that too much stress prevented LTM formation. As a control we substituted a PW bath for one of the KCl baths and LTM was still present. Thus, too much stress blocked LTM formation.

While the data from the KCl bath experiments are encouraging in that they show that the perception of stress by sensory systems can modulate memory formation, the question that must be asked is whether these data reflect what would normally occur in a snails 'everyday life'. That is, other than in *Far Side* comics, how often is a snail going to run into a KCl bath in real life? Thus, we searched for a more naturally occurring stressor. We hit on the fact that detection of a predator is a stressful phenomenon.

Crayfish effluent (CE) as a stressor

We hypothesized that our lab-reared *Lymnaea* would still respond to the scent of this predator (crayfish are sympatric predators of *Lymnaea* in The Netherlands where our snails originally came from) and that detection of this predator would significantly alter defensive vigilance behaviours. Therefore, we asked the question: when placed in a vulnerable position (i.e. with the ventral part of the foot exposed and away from the substrate), do snails alter their righting behaviour in CE? We found that snails exposed to CE significantly decreased their righting time compared with snails in pond water (PW) or boiled CE (BC) control groups. That is, when snails were placed inverted upon their shells, they took a significantly shorter time to flip over and regain their foothold on the substrate when in CE compared with PW or BC (Fig. 7).

Next we asked whether snails significantly altered their exploratory behaviour in CE, by measuring the time it took for snails to re-emerge from their shells after a small perturbation (Fig. 8, top). We exposed snails to one of the three treatments (PW, CE, BC) for 2 h then removed them from the water and placed them into a Petri dish filled with PW. The time from when the snail was first placed in the Petri dish until the snail began to crawl on the substrate was recorded. We found that snails exposed to CE took significantly longer to begin to explore their new environment compared with snails exposed to PW or BC. Thus, snails exposed to CE were more hesitant to leave the 'safety' of their shells and begin to explore their new environment.

A defensive behaviour in *Lymnaea* that has received little recent attention is the shadow-induced pneumostome withdrawal response. When *Lymnaea* are at the surface performing aerial respiration, extra-ocular photoreceptors in the pneumostome area mediate a pneumostome closure response when a shadow passes over the opened pneumostome (Stoll, 1973). We hypothesized that this defensive behaviour would be altered by exposure of *Lymnaea* to CE. Snails were placed in 500 ml of hypoxic CE or PW and allowed to acclimate, after which the shadow treatment began (Fig. 8, bottom). We found that snails in CE elicited a full pneumostome withdrawal more often when presented with a passing shadow than did snails in PW. Thus, CE exposure (i.e. detection of a predator) enhanced this defensive withdrawal response.

Collectively these data demonstrate that lab-reared *Lymnaea* are capable of detecting the presence of a crayfish predator (i.e. CE is detected) and responding in an appropriate manner. Predator detection significantly altered defensive vigilance behaviours (for details, see Orr et al., 2007).

We next determined whether aerial respiratory behaviour was altered in *Lymnaea* exposed to CE (Fig. 9). Previous reports indicate

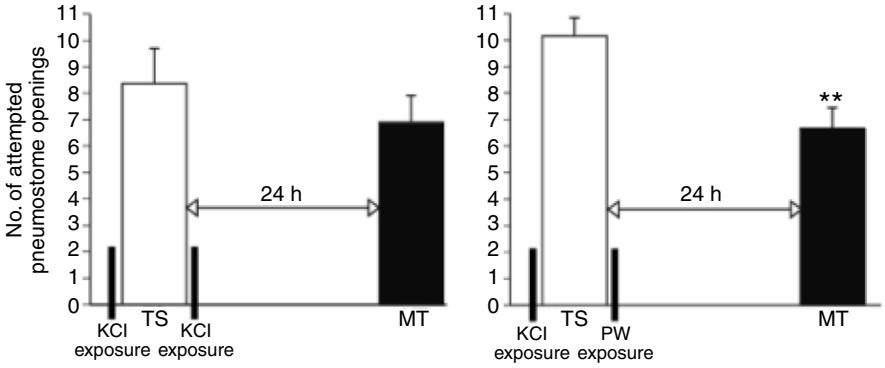

Fig. 6. Too much stress and LTM formation. Snails that received a 30–35 s KCl bath before 30 min of tactile training, and then another 30–35 s KCl bath afterwards, did not have a significant decrease in pneumostome openings in a MT 24 h later. When snails had the KCl bath and then training, but had the second KCl bath replaced with exposure to pond water (PW) there was memory, i.e. there was a significant decrease in attempted openings, 24 h later (**P<0.01). [Reprinted from Martens et al. (Martens et al., 2007a), with permission from Elsevier.]

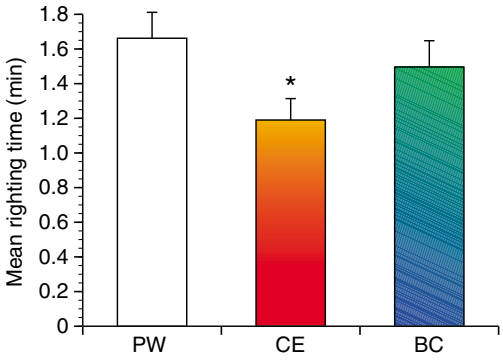

Fig. 7. Crayfish effluent detection alters the righting response in *Lymnaea*. The change in mean (±s.e.m.) righting response time after exposure to pond (PW), crayfish (CE) or boiled crayfish effluent (BC) water (N=36). PW and BE means are not significantly different from each other (P>0.05) but are significantly different (*P<0.05) from CE treatment (repeated measures ANOVA) (from Orr et al., 2007).

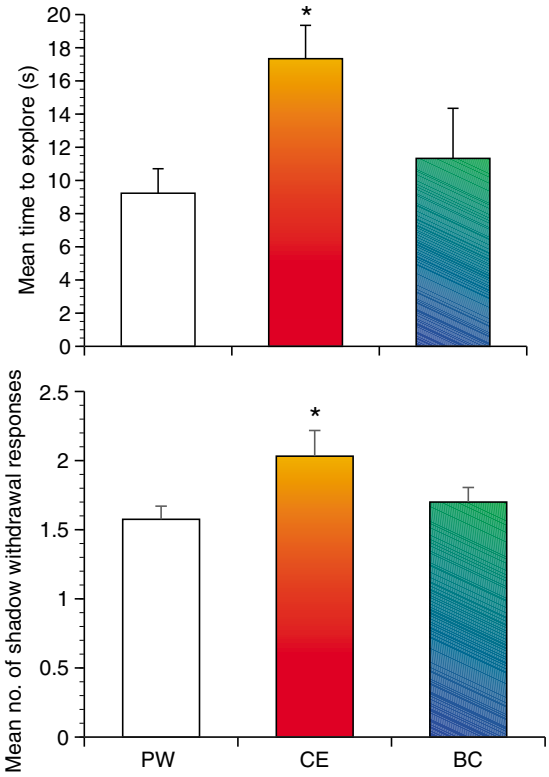

Fig. 8. CE exposure, time to explore and shadow responses. Top, the mean (±s.e.m.) time to explore for snails placed in PW after a 2 h exposure to PW, CE or BC. Time to begin to explore in the CE treatment was significantly longer compared with snails in PW and snails in BC treatment (N=54, *P<0.001, one-way ANOVA). Bottom, snails in CE elicited full pneumostome withdrawal significantly more often when presented with a passing shadow than did snails in PW or BC (from Orr et al., 2007).

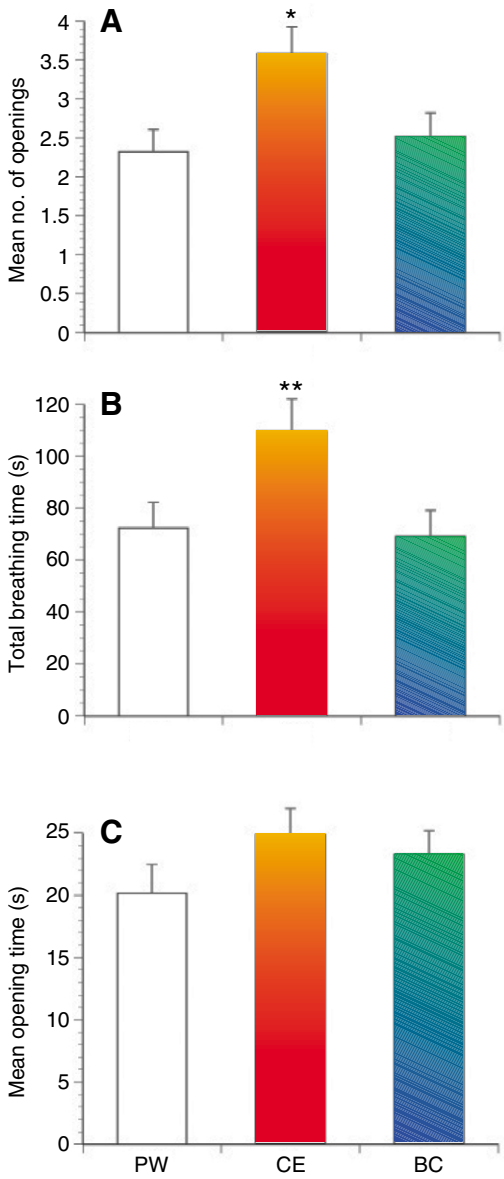

Fig. 9. Exposure to CE and aerial respiratory behaviour. The mean (±s.e.m.) number of pneumostome openings (A), total breathing time (B) and mean breathing time (C) of snails in each of the three water treatments. (A) Number of pneumostome openings in PW compared with that in CE and BC. The number for CE is significantly higher (*P<0.01, N=65) than that for either PW or BC, which were not significantly different from each other. (B) The total breathing time in PW, CE and BC (N=65). Again, CE results were significantly higher (**P<0.001) than those for either PW or BC, which were not significantly different from each other. (C) The mean breathing time was not significantly different in any of the groups (from Orr et al., 2007).

that when pulmonate snails are in the presence of a crayfish predator they tend to spend more time near the surface of the water (Dalesman et al., 2006; Turner et al., 2000; Turner and Montgomery, 2003). Presumably this is another defensive behaviour as crayfish are 'bottom' dwellers and if the snail tends to stay at the surface it would be less likely to be preyed on. We further hypothesized that if *Lymnaea* spent more time at the air–water interface as a result of CE detection, they may show a significant alteration in aerial respiratory behaviour. We therefore measured the number of pneumostome openings and the total breathing time in PW, CE and BC. These data are plotted in Fig. 9. The number of pneumostome openings (Fig. 9A) and the total breathing time (Fig. 9B) were significantly increased in CE (P<0.01, N=65) compared with controls. Interestingly, the mean breathing time for each pneumostome opening was not significantly different as a result of CE exposure (Fig. 9C, P=0.144, N=65). We conclude that these

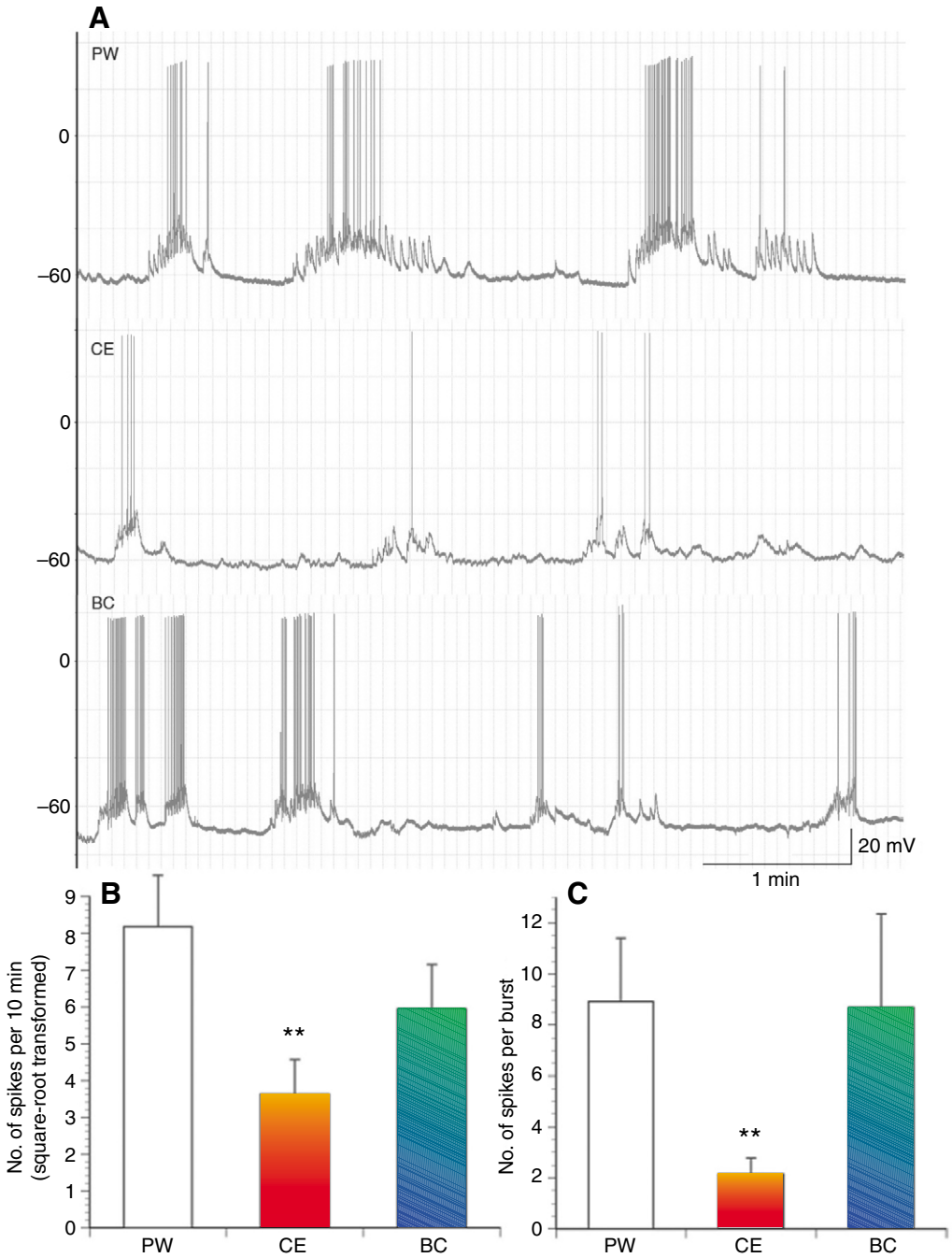

Fig. 10. CE exposure and RPeD1 activity in semi-intact preparations. (A) Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken after intact snails were exposed to PW (top), CE (middle) or BC (bottom) treatments. All traces demonstrate spontaneous firing activity, and bursting activity. (B) Summary data for mean (±s.e.m.) spiking activity per 10 min (square-root transformed, N=14). Results for CE were significantly lower (**P<0.001, N=14) than those for either PW or BC, which were not significantly different from each other. (C) Mean (±s.e.m.) number of spikes per burst. Again, results for CE were significantly lower (**P<0.001, N=14) than those for either PW or BC, which were not significantly different from each other (from Orr et al., 2007).

laboratory-reared snails are capable of detecting CE (which in the wild would signal that a crayfish predator is somewhere in the area), migrate to the air–water interface and increase the number of times they open their pneumostome to breathe, and thereby increase the total breathing time when exposed to CE.

CE and electrophysiological activity of RPeD1

As can be easily seen above, predator detection (i.e. placing snails in CE) alters a range of behaviours that possibly lessen the probability of being preyed on. We next wished to determine whether this predator detection would be reflected in a change in electrophysiological activity of a key neuron that is involved in the mediation of aerial respiratory behaviour. It has previously been found in *Aplysia* that there is often a lack of correlation between the activity of a neuron that is involved with a peripheral structure (e.g. the gill) and the behaviour of that organ (Colebrook and Lukowiak, 1988). Thus, we examined the activity of RPeD1 in semi-intact preparations that had previously been treated with PW, CE

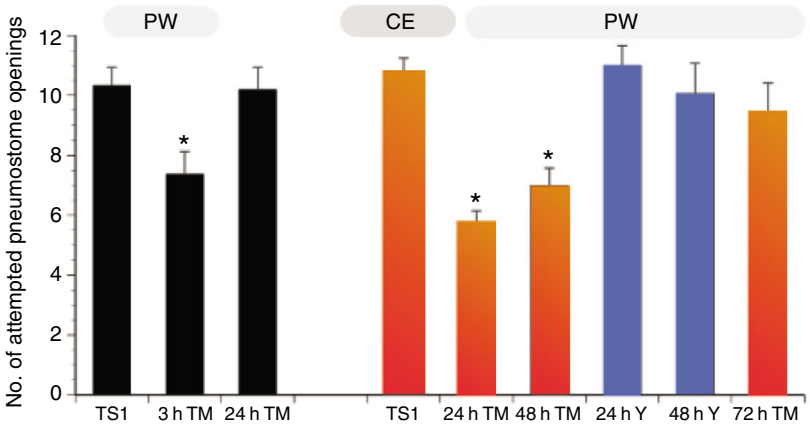

Fig. 11. Behavioural data of intact *Lymnaea* after the single 0.5 h training procedure in either PW or CE. The single training session (TS1) in PW (black bars) results in a 3 h memory (3 h TM; intermediate-term memory, ITM) but does not result in LTM (i.e. a memory lasting 24 h; black bars, N=44, P>0.05). However, the single training session (TS1) in CE (orange) results in LTM. That is, the number of attempted pneumostome openings in the memory test sessions (TM) at 24 and 48 h is significantly lower than in TS1 (i.e. memory at 24 and 48 h; 24 h TM, N=35, *P<0.01; 48 h TM, N=41, *P<0.01). Snails did not demonstrate memory formation in 24 and 48 h yoked control groups (blue bars; N=30, P>0.05 and N=20, P>0.05 for 24 and 48 h yokes, respectively) or 72 h after training (N=22, P>0.05). [Reproduced with permission from Orr and Lukowiak (Orr and Lukowiak, 2008).]

or BC (Fig. 10). We chose to examine RPeD1 because this neuron initiates the rhythmic activity that drives aerial respiratory behaviour, it receives sensory input from the pneumostome area and it is inhibited during the defensive full-body withdrawal response.

We found significant reductions in three measures of activity in the CE-treated animals compared with those treated with PW or BC (Fig. 10). The spontaneous firing activity, bursting activity and number of spikes per burst in CE-treated snails were significantly reduced compared with those in the PW- and BC-treated snails. Representative samples of recordings taken from RPeD1 in PW, CE and BC are presented in Fig. 10A, whilst summary data are presented in Fig. 10B,C. Thus, exposure of the intact snail to CE for 2 h before dissection was sufficient to significantly alter on-going electrical activity in RPeD1. We found no significant differences in other electrophysiological parameters such as resting membrane potential, action potential amplitude, duration or after-hyperpolarization.

We next asked what would happen to the snails' ability to form LTM following exposure to CE. We first examined what effect, if any, training in CE would have on memory formation when snails were subjected to a single 0.5 h training session. A naive cohort of snails was given this training in PW and then tested 24 h later (Fig. 11). As already demonstrated (Lukowiak et al., 2000; Taylor and Lukowiak, 2000; Lukowiak et al., 2003a; Lukowiak et al., 2003b; Rosenegger et al., 2004; Parvez et al., 2006a), these snails did not exhibit memory when tested 24 h later. We then asked whether similar training of snails in CE would result in augmented memory and, if so, how long would the memory persist? To answer this question, a new cohort of snails was given training in CE water and tested for memory (in PW) 24, 48 and 72 h later. Yoked controls were also performed in CE and tested in PW. To our amazement snails exposed to CE during training exhibited LTM when tested 24 and 48 h later but not at 72 h (Fig. 11). That is, the mean number of attempted pneumostome openings was significantly decreased 24 and 48 h after training but not at 72 h. In yoked control experiments in CE there was no statistical difference between training and memory test sessions. Thus, we conclude that a single 0.5 h training session in CE is sufficient to cause LTM formation that persists for at least 48 h.

Knowing that exposure to CE alone produces an effect on RPeD1 that lasts 2 h (Fig. 10) but not 24 h (Orr and Lukowiak, 2008), we next asked whether the electrophysiological profile of RPeD1 in snails subjected to the 0.5 h training session in CE was also altered. We therefore examined the properties of RPeD1 48 and 72 h after training in CE. As in the behavioural experiments we also examined a yoked control group at 48 h. We found that snails trained in CE demonstrated significantly reduced spontaneous firing frequency (measured by the number of spikes per 600 s), spikes per burst and burst duration in the 48 h operantly conditioned group, but not in the 48 h yoked or the 72 h operant groups (Fig. 12, bottom three traces and C, right three bars). From these behavioural and electrophysiological data, we conclude that exposure to CE during the 0.5 h training session enhances LTM formation. These data also suggest that for predator-induced enhancement of LTM, the electrophysiological changes in RPeD1 associated with these memories parallel the duration of the behavioural phenotype.

The data presented here are all consistent with the hypothesis that the perception of an acute stressor significantly alters adaptive behaviour. In the examples shown with both the noxious KCl stressor and CE exposure, LTM formation was significantly enhanced; and with perception of a potential predator various defensive behaviours were also altered. Finally, we were able to demonstrate neural correlates of these stress-modulated behaviours.

Because memory is a dynamic process, it is modifiable (Lukowiak et al., 2007). We first showed that the perception of a noxious stressor (the KCl bath) that elicits the whole-body withdrawal response enhances LTM formation. Here we have only focused our attention on the effects of the noxious KCl stressor but it needs to be emphasized that Martens et al. (Martens et al., 2007b) also show that similar results could be obtained using a more behaviourally relevant stimulus, namely garlic. That the perception of garlic would alter adaptive behaviour of a snail is a phenomenon that certainly deserves more thorough study!

KCl's enhancing effect on LTM formation appears to be mediated *via* sensory pathways, and not *via* a direct result of a physical action on the CNS. By that we mean that an increase in $[K^+]_o$ in the snails haemolymph as a result of K^+ diffusing across the skin of the snail while it sits in the KCl bath as the cause of LTM enhancement seems highly unlikely. If this was indeed the case then we should have seen an enhancement of memory in the yoked control experiment or when we challenged snails with a change of context test, which we did not. Moreover, we did not see an enhancement of memory when we used 25 mmol l^{-1} NaCl as a possible stressor. Thus, an as yet unidentified sensory pathway was stimulated by the KCl, and activation of this pathway resulted in the whole-body withdrawal response and enhancement of LTM formation.

Although the literature describing the effect of stress (both physical and emotional) on memory is extensive, there are few examples of stress modulating adaptive behaviours in a relatively simple invertebrate model such as ours. Furthermore, this is the first example that we are aware of in an invertebrate model system where

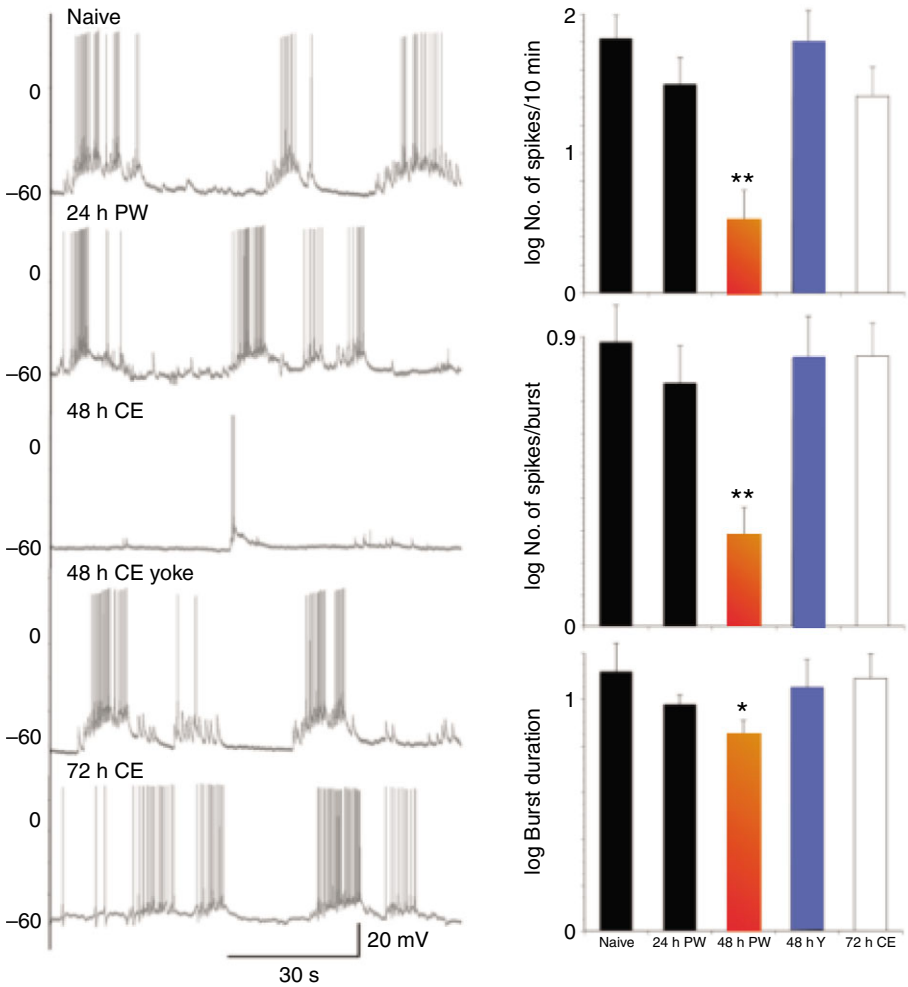

Fig. 12. Representative electrophysiological recordings of RPeD1 from semi-intact animals after the single 0.5 h training session in either PW or CE. Representative recordings from RPeD1 in the naive state (untrained and in PW), 24 h post-PW single training session, 48 h after single training session in CE, 48 h CE single training session yoke control and 72 h after the single training session in CE. Right panels: top, summary data for mean (±s.e.m.) spiking activity per 600 s; middle, number of spikes per burst (all values log transformed). Results for the 48 h single training session procedure in CE are significantly lower than the naive state (N=7, $^{**}P$<0.01). Results for 24 h single training session in PW trained animals, 48 h single training session in CE yoked and 72 h single training session in CE trained animals are not significantly different from the naive state (24 h PW single training session, N=8, P>0.05; 48 h CE single training session yoked, N=8, P>0.05; and 72 h CE single training session, N=6, P>0.05). Bottom bar graph demonstrates summary data for burst duration (mean ± s.e.m., values log transformed) of each treatment. Burst duration for single training session in CE at 48 h is significantly lower than in the naive state (N=7, P<0.05). Single training session in PW at 24 h, single training session in CE at 48 h yoked and single training session in CE at 72 h are not significantly different from the naive state (24 h PW single training session, N=8, P>0.05; 48 h CE single training session yoked, N=8, P>0.05; and 72 h CE single training session, N=6, P>0.05). [Reproduced with permission from Orr and Lukowiak (Orr and Lukowiak, 2008).]

stress has caused a significant enhancement in LTM memory formation. Moreover, since LTM of the behaviour studied here has been shown to require the molecular processes in RPeD1; we are in an excellent position to begin a direct investigation into the specific alterations in this neuron that are correlated with, if not cause, this phenomenon.

As we have stated previously, even in the laboratory, let alone in its natural environment, the probability of a snail encountering a KCl bath in its everyday existence is pretty low. However, in its natural environment the snail has a reasonably high probability of either sensing or coming into direct contact with a predator, such as a crayfish. Thus, we were motivated to determine whether lab-reared snails would still have the ability to both recognize and respond appropriately to the presence of the predator or the scent of the predator. As we have earlier reported (Orr et al., 2007) and have shown here, laboratory-reared *Lymnaea* (some 250 generations since the early 1950s without contact with crayfish) have maintained their capacity to detect prey *via* a kairomone, as evidenced by significant changes in their defensive behaviours and changes in electrophysiological parameters in a key neuron, RPeD1. We have shown that *Lymnaea* significantly increase aerial respiratory activity (which occurs when snails are at the air–water interface) when exposed to the effluent of *Procambarus clarkii* scent (i.e. CE; Fig. 4). This finding is consistent with the observation that snails often crawl out of the water when presented with shell-crushing predators such as crayfish (Alexander and Covich, 1991; Levri, 1998; McCarthy and Fisher, 2000; Turner, 1997). However, we did not detect any change in heart rate for snails exposed to CE (Orr et al., 2007), suggesting that the increase in aerial respiratory behaviour may simply be a result of spending more time near the air–water interface and not due to increased respiratory demand.

Our data suggest that once snails detect the presence of a predator, here by sensing CE, they 'decide' to alter their behavioural activities in a manner that would prove beneficial to survivorship (i.e. 'keeping a low profile' or getting out of harm's way quicker). In other words, on detecting the presence of a predator they make a risk assessment and take the appropriate actions to reduce that risk. This is not surprising given that predator detection *via* kairomones not only gives information regarding predator presence but also potentially gives information regarding the proximity, physiological state and even diet of potential predators (Dalesman et al., 2006; Kats and Dill, 1998; Wisenden, 2000). Interestingly, this 'choice' is reflected in significant alterations in the activity of RPeD1, a key neuron in the mediation of memory formation.

Exposure to CE caused a significant increase in both the 'righting' and the shadow response compared with controls (Fig. 8). These data show that snails actively reduce the duration of vulnerability on perceiving the presence of a predator. Increases in anti-predator responses when the presence of the predator is perceived have been demonstrated in many aquatic organisms in which prey respond appropriately to factors such as predator density (Wiackowski and Staronska, 1999), distance (Turner and Montgomery, 2003), size of predator and prey vulnerability (Alexander and Covich, 1991; Cotton

et al., 2004; Dewitt et al., 1999). *Lymnaea* are capable of altering their defensive responses appropriately according the perceived predator threat. That is, depending on 'who' the predator is (e.g. fish *vs* crayfish), a different defensive strategy will be taken. Differential habitat use under multiple predator systems demonstrates both increased vigilance and the differentiation between predator threats, allowing for functional tradeoffs in predator defence (Dalesman et al., 2006; Dewitt and Langerhans, 2003; Orr et al., 2007).

Here we have shown that in addition to behavioural changes brought about by CE exposure, significant changes in the electrophysiological activity in RPeD1 were also observed. For example, naive snails exposed to CE before dissection showed a significant decrease in spontaneous firing activity and bursting activity compared with control snails (Fig. 10). To our knowledge this investigation provides the first evidence of neurobiological changes associated with predator detection in pulmonates. RPeD1 has been shown to be both necessary and sufficient to drive the aerial respiratory behaviour of *Lymnaea* (Syed et al., 1990; Syed et al., 1992) and is inhibited by the defensive full-body withdrawal behaviour (Inoue et al., 1996). It is therefore not too surprising that the activity pattern of this neuron is altered when the crayfish predator is detected. However, the data presented in Figs 9 and 10 appear to be contradictory. That is, CE exposure results in a significant increase in total breathing time and the number of pneumostome openings, yet CE exposure also results in a significant decrease in RPeD1 activity, the neuron that initiates rhythmogenesis within the neural circuit that drives aerial respiration. This apparent conflict may be explained by an up-regulation in the efficacy of peripheral inputs onto downstream components of the respiratory network, which would therefore require less input from RPeD1 to initiate the respiratory rhythm. Further investigation into both the location and activity of these chemosensory receptors is ongoing in our laboratory. Previously it was demonstrated that there is an age-dependent change in suppressive input from the pneumostome area to CNS neurons, such as RPeD1 (McComb et al., 2005b). Our working hypothesis is that CE is detected by sensory neurons in the pneumostome and/or osphradial ganglion and that this activity in the peripheral nervous system modulates aerial respiratory behaviours. The interaction between the central and peripheral nervous systems of molluscs, especially as regards mediation of adaptive behaviours involving respiratory organs, is complicated, interesting and controversial (Lukowiak and Colebrook, 1988; Lukowiak and Jacklet, 1972). Which neuron(s) in the *Lymnaea* CNS actually 'makes the decision' to alter both the various behaviours and activity in RPeD1 remains to be determined.

With respect to the 'Yerkes–Dotson memory curve', any predator–prey encounter where the prey is aware of a predator presence, yet escapes the interaction with its life, should fall within a range close to the 'optimal stress intensity' for memory formation and, therefore, should augment memory formation. Unfortunately, attempts to confirm this theory experimentally have yielded mixed results (see Kim and Diamond, 2002; Shors, 2004). We showed here, using snails that have not experienced a natural predator for over 250 generations, that after exposure to CE, LTM formation was significantly enhanced compared with the typical memory in PW. In PW a 0.5 h training session only results in a memory persisting for 3 h. Here, CE exposure increased the duration of memory persistence following the single 0.5 h training session to 48 h.

Neural correlates of the CE-enhanced memory were also obtained in RPeD1. We chose to record from this neuron as it is a necessary site for LTM formation, memory reconsolidation, extinction and forgetting (Scheibenstock et al., 2002; Lukowiak et al., 2003a; Sangha et al., 2003a; Sangha et al., 2003b; Lattal et al., 2006). Moreover, we and others (Lowe and Spencer, 2006) have previously shown that significant alterations in various electrophysiological parameters in this neuron adequately reflect the significant behavioural changes that occur following training in either intact or initially naive semi-intact preparations. Thus, we were not too surprised to find that the enhanced LTM caused by training in CE was reflected in altered RPeD1 activity. Our working hypothesis is that CE exposure alters the molecular machinery in neurons (e.g. RPeD1) that are responsible for forming and maintaining the memory.

The identity of the component(s) of the stress response responsible for memory enhancement is presently unknown but previous investigations have highlighted a number of stress hormones and neural modulators that are expressed in molluscs and have been implicated in modulating memory. Our present working hypothesis, based on very preliminary data, is that serotonin plays a major role in the mediation of stress in *Lymnaea*. Our model system is very tractable in determining whether this transmitter/modulator is necessary for the stress-induced changes in adaptive behaviour since it is relatively easy to alter the serotoninergic tone of the *Lymnaea* CNS by injecting serotonin precursors, blockers or toxins that alter the serotonin content in a predictable fashion (e.g. Gadotti et al., 1986).

Our data unequivocally show that detection of a predator, an instinctive behaviour, has been maintained in our lab-reared snails over many generations and may allow us to determine at the neuronal level how such instinct is both mediated and maintained. These behaviours are robust and repeatable in the laboratory setting and support both laboratory and field investigations demonstrating that *Lymnaea stagnalis* not only detect predator kairomones but also respond in the appropriate manner to decrease the probability of predation.

Together, all the data presented above demonstrate causal links between ecologically relevant (some more than others) behaviours and neural substrates driving these behaviours. We are now beginning experiments to examine whether CE will alter behaviours in populations of *Lymnaea* where crayfish are not sympatric predators. That is, crayfish are not historically present in Alberta yet *Lymnaea stagnalis* are. Will these Alberta *Lymnaea* respond to CE in the same manner as our lab-bred snails? For that matter, will freshly collected snails from The Netherlands ('wild' Dutch snails) respond in the same manner to CE as their lab-bred cousins? Crayfish are sympatric predators of *Lymnaea* in The Netherlands. Thus, it may be possible to study at the neuronal level in a neuron such as RPeD1, which plays a necessary role in memory formation, reconsolidation, extinction and forgetting (for reviews, see Lattal et al., 2006; Parvez et al., 2006b), how an ecologically relevant stress stimulus that has been maintained in laboratory-rearing conditions affects learning and memory.

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