The Journal of Experimental Biology 213, 3603-3614

doi:10.1242/jeb.048256

# The role of serotonin in the enhancement of long-term memory resulting from predator detection in *Lymnaea*

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Accepted 5 August 2010

## SUMMARY

**Serotonergic systems play important roles in modulating stress-induced arousal and vigilance behaviours. The pond snail, *Lymnaea*, shows multiple defensive vigilance behaviours in response to the stress associated with predator detection. Predator detection elicited by crayfish effluent (CE), increases the time to re-emerge from the shell and enhances the shadow withdrawal response. More importantly, in *Lymnaea*, CE enhances the ability to form long-term memory (LTM). We investigated the role of the serotonergic system in these anti-predator responses in *Lymnaea*. Using a serotonin-receptor antagonist, mianserin, we found that two defensive vigilance behaviours (e.g. increasing the time to re-emerge from their shell and shadow response) elicited by CE were not observed when the serotonergic system was disrupted. Also, methysergide, another serotonin antagonist, blocked the enhanced LTM formation after training in CE. Importantly, mianserin did not alter LTM formation in pond water (PW). These data suggest that a serotonergic system is activated only when *Lymnaea* detect a predator. When snails were trained in CE using a training procedure that in PW produces a 24-h LTM, a more persistent form of LTM (5 days) occurred. This more persistent form of LTM was abolished after mianserin treatment. Increasing 5-HT levels in the snail by the injection of 5-HT was also associated with enhanced LTM formation. Lastly, we tested whether the osphradium is implicated in CE detection and subsequent enhanced formation of LTM. Cutting the osphradial nerve to the CNS resulted in the loss of the ability to form enhanced LTM in CE. Together, these findings support the hypothesis that the serotonergic system plays a key role in modulating the predator-induced stress responses in *Lymnaea*.**

Key words: predator detection, enhancement of LTM, serotonin, vigilance behaviours.

## INTRODUCTION

Stress modulates adaptive behaviours, sometimes impairing, and at other times facilitating, learning and memory formation (Shors, 2004). Stress is any condition that necessitates physiological, psychological and/or behavioural modification that is necessary for the well-being of the organism (Selye, 1973). We use the term 'stressor' to refer to the cause of the response, while the term stress refers to the snails' response to the stressor. Depending on the specific stressor and how the stress is perceived, memory formation and/or its recall may be enhanced or impaired (de Quervain et al., 1998; Cahill et al., 2003; Joels et al., 2006; Lukowiak et al., 2008). Thus, it is not too surprising that there is uncertainty regarding the causal mechanisms by which stress alters adaptive behaviour (Kim and Diamond, 2002). However, it is instructive to remember a quote from Selye: "Everybody knows what stress is and nobody knows what it is" (Selye, 1973).

Among the neurotransmitters studied, serotonergic systems have been implicated in an animal's response to stress, especially those associated with anxiety and defensive behaviours (Hoyer et al., 2002; Weisstaub et al., 2006; Heisler et al., 2007). In particular, stress-related behavioural arousal and vigilance are known to be modulated by a serotonergic system in mammals (Amat et al., 1998b; Amat et al., 1998c). Serotonin is thought to be involved in the process of memory consolidation following one-trial avoidance and fear conditioning which often elicits an increase in anxiety (Inoue et al., 1993; Inoue et al., 1994; Izquierdo et al., 2006; Ji and Suga, 2007; Ogren et al., 2008). Studies investigating the effect of stress on long-term potentiation (LTP) have also indicated that a serotonergic system has a variety of modulatory effects on LTP depending on the learning events (e.g. stressed or non-stressed learning) (Corradetti et al., 1992; Shakesby et al., 2002; Kojima et al., 2003; Dupin et al., 2006; Vouimba et al., 2006; Ryan et al., 2008). Serotonergic systems have also been implicated in the mediation of arousal responses in molluscan models. For instance, aversive stimuli increased serotonergic activity in the *Aplysia* central nervous system (CNS) (Marinesco and Carew, 2002; Marinesco et al., 2004a). Increasing 5-hydroxytryptamine hydrochloride (5-HT) release in the *Aplysia* CNS was accompanied by an elevated heart and locomotion rate, as well as facilitation of sensory-motor neuron synapse, all defensive, arousal responses triggered by noxious stimuli (Marinesco et al., 2004b). In Pleurobranchaea and *Tritonia*, a serotonergically modulated neuronal network was also shown to contribute to alter the properties of the central pattern generator (CPG) responsible for escape swimming, a form of arousal response following a disturbance or predator detection (Katz and Frost, 1995; Katz, 1998; Jing and Gillette, 2000).

We use *Lymnaea stagnalis*, and operantly condition its aerial respiratory behaviour. This behaviour is driven by a three-neuron CPG whose sufficiency and necessity have been experimentally demonstrated (Syed et al., 1990; Syed et al., 1992). Following conditioning, intermediate-term (ITM) or long-term memory (LTM) forms (Lukowiak et al., 1996; Lukowiak et al., 1998), depending on the training procedure used (Lukowiak et al., 2000). ITM and LTM have different molecular underpinnings in that ITM requires new protein synthesis whereas LTM needs both new protein synthesis and altered gene activity (Davis and Squire, 1984; Sutton

et al., 2001; Sangha et al., 2003c). In *Lymnaea*, a single 30-min training session in pond water (PW) produces ITM that persists for only 3 h; whereas two 0.5-h training sessions separated by a 1-h interval or a single 1-h training session in PW results in LTM, which last for at least 24 h. However, various stressors alter the ability of snails to form LTM (Sangha et al., 2003a; Martens et al., 2007a; Martens et al., 2007b; De Caigny and Lukowiak, 2008; Lukowiak et al., 2008). Most importantly, we demonstrated that *Lymnaea* improve their LTM formation with exposure to the scent of a sympatric predator [crayfish; in the form of effluent (CE)] (Orr and Lukowiak, 2008). The snails also respond to CE by showing multiple 'vigilance' behaviours (Orr et al., 2007). This predator-induced behavioural arousal was reflected in a change in electrophysiological activity of a single neuron, RPeD1, a member of the CPG, that is necessary for LTM formation (Scheibenstock et al., 2002; Sangha et al., 2003c; Orr et al., 2007; Orr and Lukowiak, 2008). Thus, we have the opportunity to examine the mechanism(s) that underlie the enhancement of memory formation by predator detection at both the behavioural and cellular levels.

Kim and Diamond (Kim and Diamond, 2002) defined stress in a manner that can be broadly applied across model species. Their definition had three requirements: (1) heightened excitability or arousal; (2) the experience must be perceived as aversive; and (3) controllability, i.e. having control over an aversive experience has a profound mitigating influence on how stressful the experience feels. We define stress as a condition resulting from a stressor (e.g. predator detection) in which an individual is aroused by an aversive situation (e.g. presence of a predator) and its ability or lack thereof to control the presence or intensity of the stimulation.

Here we questioned how the stress associated with predator detection alters adaptive behaviours including the enhancement of LTM formation. It has been suggested in *Lymnaea* that serotonergic neurons exert diverse neuromodulatory effects on the various CPG networks (Yeoman et al., 1996; Kemenes, 1997; Sakharov and Tsyganov, 2000; McCamphill et al., 2008). In part, based on those previous reports, we hypothesized that exposure to CE activates the serotonergic system that plays a key role in mediating both the initiation of vigilance behaviours and the enhancement of LTM formation.

## MATERIALS AND METHODS

### Subjects

Pond snails *Lymnaea stagnalis* (L.) were reared in our snail facility at the University of Calgary from a strain of *Lymnaea* obtained from Vrije Universeit in Amsterdam. The ancestors of these snails were collected from ditches in a polder located in the province of Utrecht in the early 1950s (Orr and Lukowiak, 2008). They were maintained in eumoxic aquaria (i.e. normal $O_2$ levels; $P_{O_2}$>9975 Pa) at room temperature, 20°C, and fed lettuce *ad libitum*. Adult snails with a shell length of 2.5–3.0 cm were used in the experiments.

### Aerial respiratory behaviour

*Lymnaea* can exchange gases through its lung (aerial respiration) connected to the outside *via* the respiratory orifice (the pneumostome) as well as cutaneously across its skin. Cutaneous respiration is predominant in eumoxia; however, *Lymnaea* switch to aerial respiration to satisfy their respiratory needs in a hypoxic environment. To perform aerial respiration, snails must come to the water surface and then open their pneumostome, while, at the same time contracting and relaxing mantle muscles in order to exchange air in their lungs with atmosphere (Lukowiak et al., 2003).

### Breathing observation

To ensure that the drug (see below) or surgical treatment in these experiments did not cause any adverse effect on snails' baseline aerial respiratory behaviour, breathing observation sessions were performed. In breathing observation sessions, snails were placed in a 1 liter beaker containing 500 ml of hypoxic pond water ($P_{O_2}$<931 Pa; PW), given a 10 min acclimatization period, and their breathing behaviour was observed in a 30 min session. The time at which each snail opened and closed their pneumostome was recorded. From these data, the total breathing time, the average pneumostome opening time, and the number of openings (pneumostome) before and after drug treatments were calculated and compared to determine whether the drugs affected in any way the snail's ability to perform aerial respiration.

So far, two 5-HT receptor genes have been identified in *Lymnaea*, which are designated 5-$HT_{1Lym}$ and 5-$HT_{2Lym}$, enabling us to manipulate the serotonergic system by established agonists and antagonists (Gerhardt et al., 1996). The 5-HT receptor antagonist mianserin (obtained from Sigma-Aldrich, St Louis, MO, USA and used extensively in many molluscan studies) and methysergide (Sigma-Aldrich) were dissolved in normal *Lymnaea* saline (composition in mmol $l^{-1}$: 51.3 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, 5.0 Hepes, pH 7.9). The solutions were injected into the haemocoel through the foot of the snails. The concentrations and injection time was determined and optimized based on our pilot data from breathing observation studies. We chose to use 7.5 μg $ml^{-1}$ mianserin and 0.94 μg $ml^{-1}$ methysergide. These drugs were injected 2.5 h before first training session in all experiments. To confirm whether the increase of serotonergic activity was indeed involved in the enhanced LTM, 5-HT (Sigma-Aldrich) dissolved in saline was also used. We injected 10.63 μg $ml^{-1}$ 5-HT 3 h before a single 30-min PW training based on our pilot data.

### Operant conditioning

Snails were individually labelled and placed in a 1 litre glass beaker containing 500 ml of PW or CE made hypoxic ($P_{O_2}$<931 Pa) by bubbling $N_2$ through it for 20 min prior to, and during, each training and test session. In the hypoxic environment, *Lymnaea* significantly increase their respiratory behaviour (Lukowiak et al., 1996). Snails were given a 10-min acclimatization period and allowed to freely perform aerial respiration. At the onset of the training or test session snails were gently pushed beneath the water surface. In the training sessions the snails were operantly conditioned by applying a gentle tactile stimulus with a sharpened wooden applicator to their pneumostome whenever they attempted to open it. The number of attempted pneumostome openings was recorded and the time at which a snail received the tactile stimulus was recorded for the yoked control experiments (see below). Between training and test sessions, the snails were returned to their home eumoxic aquaria.

Snails were subjected to one of the following training procedures: (1) a single 30-min training session; (2) two 30-min training sessions separated by a 1 h interval; and (3) a single 1-h training session in PW or CE. A memory test session was performed at the following times: (1) 3 h later to determine if ITM was present; (2) 24 h or more (e.g. 5 days) to test if LTM was present. All memory test sessions were performed in PW.

To obtain CE, 500 ml of water was taken from aquaria where crayfish are maintained.

### Yoked control

Yoked control animals underwent the same experimental treatment, with one exception. For the yoked control training session, the snails

received exactly the same number of stimuli using the same pattern of stimulation as those of the operant conditioning group. That is, they received the tactile stimulus when the snail to which they were 'yoked' to opened its pneumostome in the operant conditioning procedure. Thus, the presentation of the tactile stimulus was not contingent on an attempted pneumostome opening of the yoked control snail. In the memory test session, these snails received the tactile stimulus to the pneumostome when they attempted to open their pneumostomes.

### The exposure to crayfish effluent and vigilance behaviours

To test the effect of the drug treatment on snails' vigilance state, we analyzed two behaviours: re-emerging and shadow withdrawal. To examine re-emerging behaviour, snails were placed in a 1 litre beaker filled with 500 ml PW or CE for 1 h before the behavioural trial. Snails were taken from the beaker and immediately placed into a 9 cm Petri dish filled with PW. This procedure was shown to evoke the full-body withdrawal behaviour in the snails (Orr et al., 2007). We then measured the time it took for the snail to re-emerge from its shell (i.e. determined when the snail extended its tentacles and its head could be completely observed). This procedure was performed five times for each snail and the mean ± standard error of the mean (±s.e.m.) were calculated.

To test the shadow withdrawal behaviour, snails were placed in a 1 litre beaker containing 500 ml hypoxic water (PW or CE) and allowed to acclimate for 1 h. A 35 W halogen light, 50 cm above the beaker, was turned on and a shadow was passed over the snails, using a 2 cm diameter piece of cardboard attached to the end of a stick, whenever the snails attempted to perform aerial respiration. We measured the number of shadow-induced withdrawal responses before and after drug treatments. Each snail was tested five times and the mean ± s.e.m. was calculated for each treatment.

### Surgical procedure

The osphradium of *Lymnaea* processes chemosensory information (Wedemeyer and Schild, 1995). As a potential locus for perceiving CE, we focused on the osphradium since 5-HT neurons were found and 5-HT was involved in the chemosensory information processing in this organ (Nezlin et al., 1994; Kamardin et al., 1999). Briefly, the animals were anaesthetised using iced pond water and 2 ml of 50 mmol $l^{-1}$ $MgCl_2$ were injected into the haemocoel through the foot. Anesthetized animals were placed in a dissection dish and the osphradial nerve innervating the CNS was cut proximal to the osphradium through a small opening in the skin. Sham-treated animals were prepared as a control group using an identical procedure, but only a very small incision was made above the osphradial nerve leaving the nerve intact. All animals were given 3 days to recover from the surgery.

### Criteria of memory in this experiment

Memory was considered to be present if the number of attempted pneumostome openings in memory test (MT) session was significantly lower than that of the first training session (TS1) and was not significantly higher than that of last training session (TS2).

### Statistics

To determine any effects of the drug treatment on the snails' basal aerial respiratory behaviour [i.e. between before the breathing observation (preBO) and postBO] and memory formation (i.e. between TS and MT), paired Student's *t*-test (between groups) was conducted. A repeated measures analysis of variance (ANOVA) was performed followed by a Tukey–Kramer *post hoc* test for experiments that involved three or more measures in the same group if the data pass the normality test. If the data did not pass the normality test, we analyzed the data with a Friedman's test followed by a Dunn's multiple comparison *post hoc* test. When groups were not matched, a Kruskal–Wallis (KW) test was conducted followed by a Dunn's multiple comparison *post hoc* test. To compare the number of attempted pneumostome openings between TS and yoked control or between sham-operated and osphradial nerve cut animals, unpaired *t*-test was used. In all cases, differences were considered to be significant if $P<0.05$ (*) or $P<0.01$ (**).

## RESULTS

### The effect of serotonin receptor antagonists and 5-HT on breathing behaviour

We first determined the effect of serotonin antagonists on aerial respiratory behaviour. We measured the total breathing time, the average pneumostome opening time, and the number of pneumostome openings in a 30-min observation session before and after drug treatment. Two naïve cohorts of snails were observed in a breathing observation session 24 h before mianserin ($N$=12) or methysergide ($N$=10) injection (preBO). Next, 7.5 μg mianserin $ml^{-1}$ or 0.94 μg methysergide $ml^{-1}$ were injected into the hemocoel of each snail through the foot, 2.5 h before the second breathing observation (postBO). As can be seen Fig. 1A–C (A, total breathing time; B, the average pneumostome opening time; C, the number of openings), none of the three parameters of aerial respiration were altered by either of the serotonin antagonists (paired *t*-test, all $P>0.05$). Higher concentrations of these two drugs significantly altered breathing behaviour and locomotion and were therefore not used. To test whether 5-HT significantly affects the basal breathing behaviour, a naïve cohorts of snails ($N$=10) were also subject to breathing observation (same as described above) before and after 5-HT treatment (10.63 μg 5-HT $ml^{-1}$ snail volume). 5-HT was injected 3 h before PostBO. The result showed that 5-HT injection also did not have any effects on the basal aerial respiratory behaviour in our snails (paired *t*-test; all $P>0.05$; Fig. 1A–C).

### Mianserin abolished vigilance behaviours elicited by CE

We then investigated whether the vigilance behaviours elicited in *Lymnaea* by CE would be altered by blockage of a serotonergic system. Considering the role played by serotonergic systems in behavioural arousal in both invertebrates and vertebrates (Geyer, 1996; Rueter et al., 1997; Weiger, 1997; Gillette, 2006), it is conceivable that serotonin is involved in the mediation of vigilance behaviours elicited by predator detection. We examined two of these vigilance behaviours: (1) the time it takes for snails to re-emerge from their shell following the whole body withdrawal response; and (2) the increased sensitivity of the shadow withdrawal response.

A naïve cohort of snails ($N$=13) was exposed to 500 ml PW or CE in a 1 liter beaker for 1 h then gently taken from the beaker and immediately placed into a Petri dish filled with PW. When snails were placed in the Petri dish, the whole body withdrawal response was elicited and we measured how long it took each snail to re-emerge from their shell and begin to crawl around the substratum. To test the effect of mianserin on the re-emerging behaviour, the same snails received identical treatment described above except that they were injected with either saline or mianserin 2.5 h before the test. Each treatment (PW, CE, saline+CE, mianserin+CE) was performed on successive days. Snails stayed in their shell significantly longer following CE exposure than PW. That is, CE exposure significantly increased the snails' overall time to re-emerge from their shells ($ANOVA_{(13,3)}$ $F$=12.173, $P<0.01$ for PW *vs* CE;

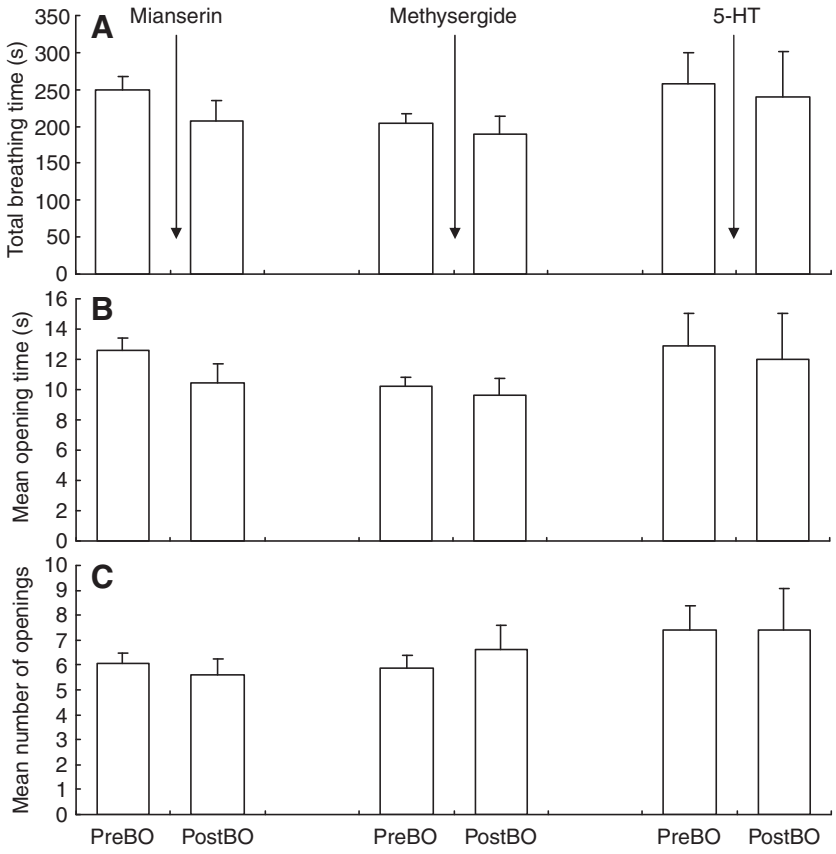


Fig. 1. Serotonin antagonists on aerial respiratory behaviour of serotonin antagonists on aerial respiratory behaviour. Each breathing observation (BO) was performed in hypoxic PW for 30 min. PreBO was performed 24 h before PostBO. 7.5 μg mianserin ml$^{-1}$ and 0.94 μg methysergide ml$^{-1}$ were injected 2.5 h before PostBO. 10.63 μg 5-HT ml$^{-1}$ was injected 3 h before PostBO (mianserin group, *N*=12; methysergide group, *N*=10; 5-HT group, *N*=10). (A) Total breathing time. There was no significant difference between preBO and postBO (paired-test, all three groups $P>0.05$). (B) The average pneumostome opening time. This was not significantly altered after each drug treatment (paired *t*-test, all $P>0.05$). (C) Mean number of pneumostome openings. No statistical difference were found between the preBO and postBO measurements in any of the treatment groups (paired-test, all $P>0.05$).

$P<0.01$ for PW *vs* saline+CE; Fig. 2A). However, when the snails were treated beforehand with mianserin, we could not observe any difference in the re-emergence behaviour following CE exposure compared with PW. The mianserin+CE treatment also significantly decreased the overall time to re-emerge compared with the CE alone and saline+CE treatments ($ANOVA_{(13,3)}$ $F=12.173$, $P>0.05$ for PW *vs* mianserin+CE; $P<0.01$ for CE *vs* mianserin+CE; $P<0.01$ for saline+CE with mianserin+CE; Fig. 2A).

Next, we determined whether blockade of the serotonergic system would alter another vigilance behaviour. The shadow stimulus causes *Lymnaea*, when they are at the air–water interface, to close their pneumostome (Stoll, 1973). This response rapidly habituates. However, the sensitivity of shadow-elicited pneumostome closure response was elevated in CE and does not habituate readily (Orr et al., 2007). Thus, we injected mianserin into our snails and observed the effect of the drug on the elevated shadow response in CE. First, a cohort of naïve snails (*N*=24) were placed in hypoxic PW or CE for 1 h. We then measured their shadow withdrawal response (see Materials and methods). Saline and mianserin were injected 2.5 h before the experiment, and each treatment (PW, CE, saline+CE, mianserin+CE) was carried out on successive days. We found that the shadow-induced pneumostome closure was more often observed in CE and saline+CE treatments than in the PW alone treatment ($ANOVA_{(23,3)}$ $F=33.377$, $P<0.01$ for PW *vs* CE; $P<0.01$ for PW *vs* saline+CE; Fig. 2B). However, this increased sensitivity of shadow response in CE disappeared after mianserin injection. That is, the shadow-induced response in mianserin+CE treatment was not significantly different from those in PW, but significantly decreased compared with the response in CE and saline+CE treatment ($ANOVA_{(23,3)}$ $F=33.337$, $P>0.05$ for PW *vs* mianserin+CE; $P<0.01$ for CE *vs* mianserin+CE; $P<0.01$ for saline+CE *vs* mianserin+CE; Fig. 2B). These data are all consistent with the hypothesis that the vigilance behaviours induced by CE exposure are mediated *via* a serotonergic system.

### Serotonin receptor antagonist mianserin did not block LTM in PW

Having shown that serotonin plays a significant role in mediating vigilance behaviours elicited by CE, we hypothesized that 5-HT plays a role in the CE-induced enhancement of LTM formation. However, before we could determine what role 5-HT plays in CE-induced enhancement of LTM we first needed to determine whether blocking 5-HT altered LTM formation in PW.

Three cohorts of naïve snails (no-drug group, *N*=14; saline group, *N*=12; and mianserin group, *N*=14) were trained in PW (two 0.5-h training sessions separated by a 1-h interval) and memory was tested 24 h later. LTM was present in all three groups (Fig. 3). That is, the number of attempted pneumostome openings were significantly decreased in a 24-h memory test (MT) compared with training session 1 (TS1) in all groups (no-drug group, $ANOVA_{(13,2)}$ $F=22.528$, $P<0.01$; saline group, $ANOVA_{(11,2)}$ $F=11.619$, $P<0.05$; mianserin group, $ANOVA_{(13,2)}$ $F=17.815$, $P<0.01$; Fig. 3). In addition a yoked control mianserin-injected group was used. This group showed no decrease of the number of attempted pneumostome openings in the 24-h MT compared with those of mianserin-injected group on TS1 (unpaired *t*-test, $P>0.05$; Fig. 3). Thus, mianserin does not block LTM formation resulting from a training procedure in PW.

### Serotonin receptor antagonist mianserin blocked the enhanced LTM in CE

Having shown that LTM in PW is not blocked by mianserin, we investigated whether mianserin blocks the enhancement effect of CE on LTM (Fig. 4). Three cohorts of naïve snails (no-drug group, *N*=14; saline group, *N*=17; and mianserin group, *N*=16) were

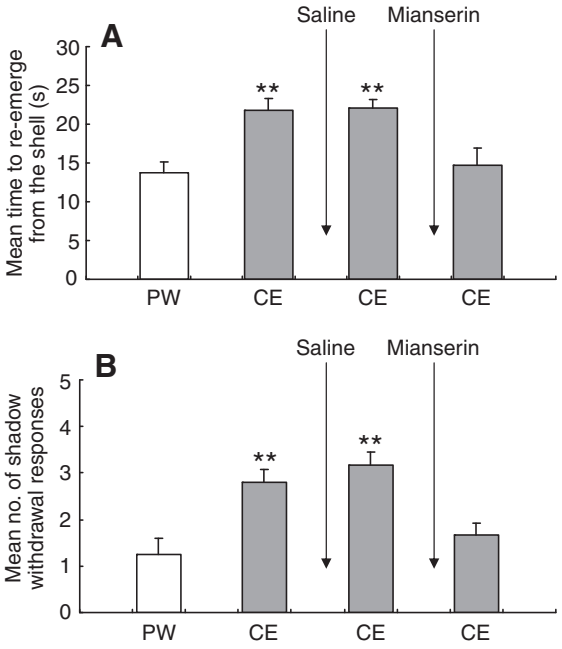


Fig. 2. Mianserin abolished the snails' vigilance behaviours in CE. (A) The mean time to re-emerge from shell after small perturbation. Snails ($N$=13) were subject to PW, CE, saline+CE, and mianserin+CE treatments on alternate days. The overall time until the snails re-emerged from their shells was significantly increased in the CE and saline+CE treatments compared with the PW treatment ($ANOVA_{(13,3)}$ $F$=12.173, $P$<0.01 for PW *vs* CE; $P$<0.01 for PW *vs* saline+CE). However, in the mianserin+CE treatment, the snails did not show any significant increase in the overall time to re-emerge compared with the PW alone treatment, and the overall time to re-emerge from shell was significantly decreased compared with the CE alone and saline+CE treatments ($ANOVA_{(13,3)}$ $F$=12.173, $P$>0.05 for PW *vs* mianserin+CE; $P$<0.01 for CE *vs* mianserin+CE; $P$<0.01 for saline+CE *vs* mianserin+CE). (B) The mean number of shadow-elicited full pneumostome withdrawals. Snails ($N$=24) in the CE and saline+CE treatments more often closed their pneumostomes and withdrew into their shells when presented with a shadow passing over their body than they did in the PW treatment ($ANOVA_{(23,3)}$ $F$=33.377, $P$<0.01 for PW *vs* CE; $P$<0.01 for PW *vs* saline+CE). However, in the mianserin+CE treatment, the snails did not show an increase in the number of full pneumostome withdrawals compared with the PW treatment and they decreased the number of full pneumostome withdrawals compared with CE and saline+CE treatments ($ANOVA_{(23,3)}$ $F$=33.337, $P$>0.05 for PW *vs* mianserin+CE; $P$<0.01 for CE *vs* mianserin+CE; $P$<0.01 for saline+CE *vs* mianserin+CE). PW, pond water; CE, crayfish effluent; **$P$<0.01. Grey shading signifies training in CE.

subjected to a single 30-min training session (TS) in CE. Saline and mianserin were injected 2.5 h before the single training session. The single 30-min training session in CE resulted in LTM lasting for at least 48 h, whereas the same training procedure in PW resulted in ITM lasting for only 3 h (Orr and Lukowiak, 2008). Both the non-injected group and the saline-injected group demonstrated LTM following the single 30-min training session in CE. That is, there was a significant decrease in the number of attempted pneumostome openings in the 24-h MT compared with the number of attempted pneumostome openings in the TS (paired $t$-test, both groups, $P$<0.01; Fig. 4). However, in the mianserin-injected group LTM was not observed. That is, there was no significant decrease in the number of attempted pneumostome openings in the 24-h MT compared with the number of attempted openings in the TS (paired $t$-test, $P$>0.05; Fig. 4). It should be noted that the mianserin-injected group showed a slight but not statistically significant increase in the number of attempted pneumostome openings in the TS compared with those of other two groups ($ANOVA_{(46,2)}$ KW=4.507, $P$>0.05). Thus, CE did not enhance LTM formation in the mianserin-injected snails, consistent with our hypothesis that a serotoninergic network is involved in the enhancement process.

In an additional experiment (data not shown), we injected mianserin 5 h before the single 0.5-h TS in CE. In this experiment LTM formation was also blocked, indicating that the mianserin effect remains for at least 5 h. Thus, we could confidently use mianserin to disrupt the serotonergic system in other training procedures, such as two sessions of 30 min training separated by a 1-h interval and a single 1-h training (i.e. all these training procedures were finished within 5 h).

### ITM formation is not affected by mianserin

When trained in PW a single 30 min training session results in a 3-h memory, and has been termed intermediate memory (ITM) (Sangha et al., 2003c). However, when trained in CE this training procedure results in LTM. ITM is dependent on new protein synthesis whereas both new protein synthesis and altered gene activity are necessary for LTM formation (Sangha et al., 2003c). Since snails trained in CE form LTM after a single 30-min training session, we assumed that CE exposure enhances the consolidation process necessary for LTM *via* a serotonergic pathway that ultimately leads to altered gene activity in neurones essential for memory. Thus, even if the serotonergic system is disrupted, a single 30-min training session in CE should still result in ITM because ITM formation is not dependent on altered gene activity. To test this hypothesis, a no-drug group ($N$=14), a saline-injected group ($N$=12), and a mianserin-injected group ($N$=13) were trained in CE for 30 min (TS), and subsequently tested for ITM 3 h after training (3-h MT). As predicted, all three groups exhibited ITM. That is, the number of attempted pneumostome openings in the 3-h MT was significantly lower than in the TS in all groups (paired $t$-test, all groups, $P$<0.01; Fig. 5). In yoked controls the number of attempted pneumostome openings in the 3 h MT was not statistically different from those of the mianserin-injected group in the TS (i.e. ITM was not formed; Fig. 5). Thus, ITM was not affected by the blockade of the serotonergic system in CE. These data are consistent with our hypothesis that a serotoninergic network is activated by CE and alters gene transcriptional events necessary for the enhanced LTM.

### Longer lasting LTM with different training procedures and CE enhancement of LTM

Orr and Lukowiak (Orr and Lukowiak, 2008) showed that CE lead to a more persistent LTM when they used a training procedure consisting of two 0.5-h training sessions with a 1-h interval between the training session (8 days in CE *vs* 24 h in PW). We wanted to know if mianserin would block the enhancing effect of CE with this training procedure. We tested three cohorts of naïve snails (no-drug group, $N$=36; saline-injected group, $N$=38; and mianserin-injected group, $N$=34) in two 30-min training sessions separated by an hour (TS1 and TS2) in CE. After two 0.5-h trainings in CE, some of the snails were tested for memory 24 h later (no-drug group, $N$=12; saline group, $N$=12; and mianserin group, $N$=11 for 24 h MT in PW) and the remaining snails were tested 48 h (no-drug group, $N$=12; saline group, $N$=12; and mianserin group, $N$=11 for 48 h MT in PW) and 5 days later (no-drug group, $N$=12; saline group, $N$=14; and mianserin group, $N$=12 for 5 days MT in PW). In both the no-drug group and saline-injected group, CE produced an enhanced 5 day LTM. That is, the number of attempted pneumostome openings was significantly decreased in 24 h MT, 48 h MT and 5 days MT (no-drug group; $ANOVA_{(35,4)}$ KW=50.414, $P$<0.01 for 24 h MT, $P$<0.01

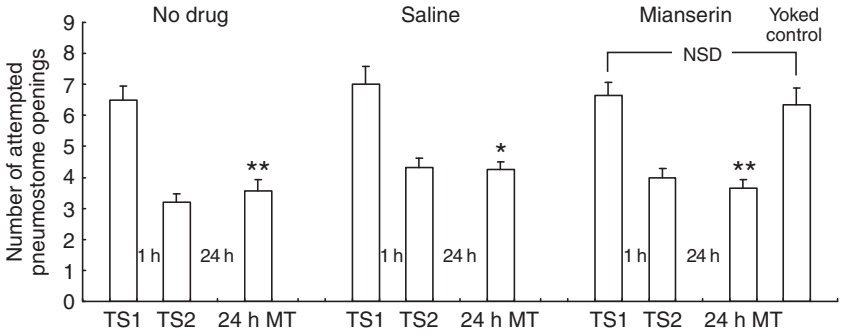


Fig. 3. Mianserin had no effect on LTM formation in PW. Each group (no-drug group, *N*=14; saline group, *N*=12; mianserin group, *N*=14) were trained in PW by using two 0.5 h training sessions separated by a 1 h interval, which typically resulted in LTM formation. Drug injection (i.e. saline and mianserin) was performed 2.5 h before the first training session. In all three groups, LTM was observed when tested 24 h later memory test session (one way ANOVA with repeated measure: no-drug group $P<0.01$, saline group $P<0.05$, mianserin group $P<0.01$). The yoked control group showed no significant decrease of the number of pneumostome openings in the 24 h MT compared with TS1 in the mianserin-injected group (unpaired *t*-test, $P>0.05$). Thus, mianserin did not block the 'normal' LTM formation in PW nor did it alter the snails' motor function to perform the aerial respiration. TS1, training session 1; TS2, training session 2; MT, memory test; NSD, no significant difference; *$P<0.05$, **$P<0.01$.

for 48 h MT, $P<0.01$ for 5 days MT, saline-injected group; $ANOVA_{(37,4)}$ KW=57.765, $P<0.01$ for 24 h MT; $P<0.01$ for 48 h MT, $P<0.01$ for 5 days MT; Fig. 6A,B). LTM was also present in the mianserin-injected group when memory was tested 24 h later following the two 0.5-h training sessions in CE ($ANOVA_{(33,4)}$ KW=33.262, $P<0.01$ for 24 h MT; Fig. 6C). Yoked control snails confirmed that the decrease of aerial respiration during the 24 h MT in the mianserin group was not the result from a possible side-effect of the drug, but from operant conditioning (data not shown). However, in the mianserin-injected group, LTM was not observed 48 h and 5 days later. That is, the number of attempted pneumostome openings in the 48 h MT ($P>0.05$) and the 5 days MT ($P>0.05$) was not significantly different from that of TS1 (Fig. 6C).

LTM persisting for 24 h is also formed in PW following a single 1-h training session (Sangha et al., 2003c). It has not yet been determined whether training in CE would enhance LTM formation following a single 1-h training session. If CE does enhance LTM following the 1-h training session, then we would have another opportunity to determine if mianserin only blocks the enhancement of memory and not the memory forming process *per se*. Therefore a cohort of naïve snails (no-drug group; *N*=35) received a single 1-h training in CE and their memory retention was tested in the following sessions: 24 h MT (*N*=10), 48 h MT (*N*=13) and 5 days MT (*N*=12). We found that the 1-h training session in CE resulted in a 5-day LTM. That is, the number of attempted pneumostome openings in 24 h MT, 48 h MT and 5 day MT was significantly lower than in TS ($ANOVA_{(34,3)}$ KW=32.112, $P<0.01$ for 24 h MT, $P<0.01$ for 48 h MT, $P<0.01$ for 5 days MT; Fig. 7A). Subsequently, both saline-injected groups (*N*=33) and mianserin-injected groups (*N*=35) were subjected to the single 1-h training session in CE and memory was tested in the following sessions: 24 h MT (saline group, *N*=10; mianserin group, *N*=11), 48 h MT (saline group, *N*=12; mianserin group, *N*=12) and 5 days MT (saline group, *N*=11; mianserin group, *N*=12), respectively. We found that LTM persisted for at least 5 days in the saline-injected snails. That is, the saline-injected groups showed LTM in the 24 h MT, 48 h MT, and 5 days MT sessions

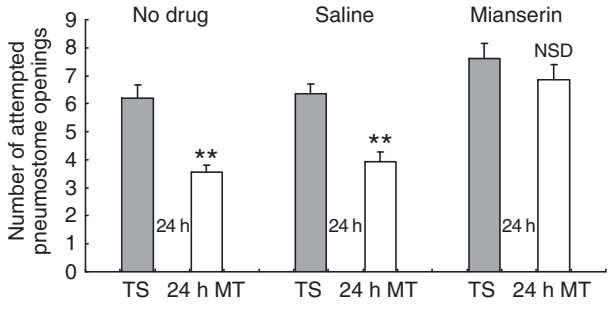


Fig. 4. Mianserin blocked the enhanced LTM formation in crayfish effluent (CE). A single 30-min training session was sufficient to produce LTM in both the no-drug (*N*=14) and saline-injected (*N*=17) snails. In both groups, the number of attempted pneumostome openings in the 24 h MT was significantly decreased compared with those of the TS (paired *t*-test, both groups; $P<0.01$). However, the mianserin-injected snails showed no memory in the 24 h MT session following training in CE (i.e. the number of attempted pneumostome openings was not significantly less than in TS; paired *t*-test, $P>0.05$). Among three groups, the number of pneumostome openings in TS was not significantly different ($P>0.05$). TS, training session; MT, memory test; NSD, no significant difference; **$P<0.01$. Grey shading signifies training in CE.

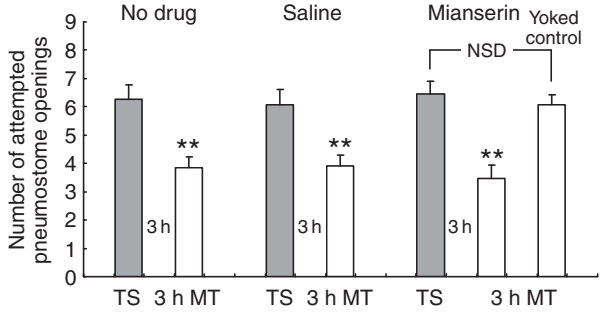


Fig. 5. Mianserin had no effect on ITM formation in crayfish effluent (CE). Three cohorts of naïve snails (the no-drug group, *N*=14; saline group, *N*=12; mianserin group, *N*=13) received a single 0.5-h training in CE and their memory was tested 3 h later. Memory was present 3 h MT in all three groups. That is, the number of attempted pneumostome openings in the 3 h MT was significantly less than in the TS (paired *t*-test, all $P<0.01$). In the yoked control experiment, mianserin did not decrease the number of attempted pneumostome openings in the 3 h MT compared with those of the mianserin group in the TS (unpaired *t*-test, $P>0.05$). NSD, no significant difference; **$P<0.01$. Grey shading signifies training in CE.

($ANOVA_{(32,3)}$ KW=25.184, all $P<0.01$; Fig. 7B). However, the mianserin-injected snails only exhibited memory in the 24 h MT. They did not exhibit memory in the 48 h or 5 days MT ($ANOVA_{(34,3)}$ $F=6.493$, $P<0.01$ for 24 h MT; $P>0.05$ for 48 h MT; $P>0.05$ for 5 days MT; Fig. 7C). These results showed that the ability to form 'normal' LTM was not impaired by mianserin in CE. However, the blockade of serotonergic activation in CE caused the snails to lose the enhanced ability of forming a more persistent LTM.

### Serotonin receptor antagonist methysergide also blocked the enhanced LTM formation

We next asked whether a different, but widely used, molluscan serotonin receptor antagonist methysergide (Walcourt-Ambakederemo and Winlow, 1994; Straub et al., 2007) blocks the enhancing effect of CE on LTM. Two cohorts of naïve snails (saline group, $N=13$; methysergide group, $N=13$) were injected with either saline or 0.94 μg methysergide $ml^{-1}$ snail volume 2.5 h before the operant conditioning training. They received a single 0.5 h training session (TS) in CE and their memory retention was tested 24 h later in PW (24 h MT). As shown in Table 1, the methysergide-injected snails did not exhibit LTM while the saline-injected snails did. That is, the number of attempted pneumostome openings in the 24 h MT was not statistically different from that of TS in the methysergide group (paired $t$-test, $P>0.05$; Table 1). Thus, the data obtained with this different serotonin receptor antagonist are similar to the data obtained with mianserin.

### 5-HT can enhance LTM formation in PW

Considering our finding that the enhancing effect on memory formation of CE was abolished by serotonin receptor antagonists,

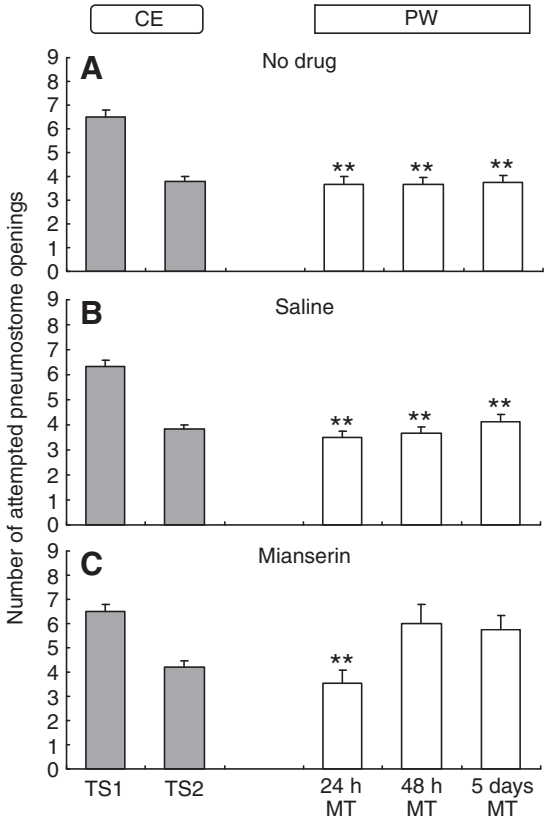


Fig. 6. A more persistent form of LTM caused by CE training is not observed after mianserin treatment. Three groups of naïve snails (no-drug, $N=36$; saline group, $N=38$; mianserin group, $N=34$) were trained in CE using two sessions of 30 min. (A,B) Both the no-drug and the saline group showed at least 5-day memory after two sessions of 30 min training in CE. That is, the number of attempted pneumostome openings were significantly decreased compared with TS1 when memory was tested 24 h (no-drug group, $N=12$; saline group, $N=12$), 48 h (no-drug group, $N=12$; saline group, $N=12$) and 5 days later (no-drug group, $N=12$; saline group, $N=14$; one-way ANOVA with repeated measures, $P<0.01$ for 24 h MT, $P<0.01$ for 48 h MT, $P<0.01$ for 5 days MT in both groups). (C) The mianserin-injected group showed a normal LTM lasting for only 24 h ($N=11$; $P<0.01$). However, the more persistent form of LTM as seen in the other groups disappeared when memory was tested 48 h ($N=11$) and 5 days later ($N=12$). That is, any significant differences in the number of attempted pneumostome openings were not detected between TS1 and 48-h/5-day MT in the mianserin group ($P>0.05$). NSD, no significant difference; $^{**}P<0.01$. Grey shading signifies training in CE.

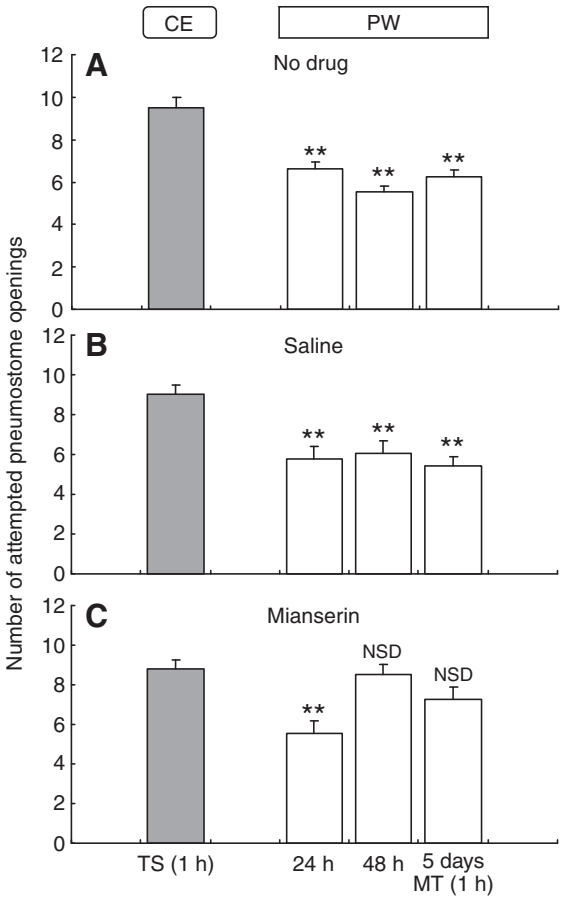


Fig. 7. A single 1 h training in CE produced LTM persisting for at least 5 days and this long lasting memory was abolished by mianserin. The no-drug group ($N=35$), saline group ($N=33$), and mianserin group ($N=35$) received a single 1-h training session in CE and their memory was tested immediately and 24 h, 48 h and 5 days later. (A) The no-drug group consistently showed LTM at 24 h ($N=10$), 48 h ($N=13$) and 5 days ($N=12$; one-way ANOVA with repeated measures, $P<0.01$ for 24 h MT, $P<0.01$ for 48 h MT, $P<0.01$ for 5 days MT). (B) The saline-injected group also showed LTM persisting for at least 5 days ($N=10$ for 24 h MT, $P<0.01$; $N=12$ for 48 h MT, $P<0.01$; $N=11$ for 5 days MT, $P<0.01$). (C) The mianserin-injected group was able to produce a normal LTM lasting for 24 h ($N=11$, $P<0.01$); however, the more persistent form of LTM was not observed at 48 h ($N=12$, $P>0.05$) or 5 days ($N=12$, $P>0.05$). NSD, no significant difference; $^{**}P<0.01$. Grey shading signifies training in CE.

Table. 1. Methysergide also blocked the enhanced memory formation caused by crayfish effluent

| Treatment | Number of attempted pneumostome openings | *P* | *N* |
|---|---|---|---|
| Saline | | | |
| TS in CE | 6.61±0.416 | 0.0007** | 13 |
| 24-h MT | 4±0.277 | | |
| Methysergide | | | |
| TS in CE | 6.38±0.474 | 0.6938 | 13 |
| 24-h MT | 6.07±0.430 | | |

Values are means ± s.e.m.
TS, training session; MT, memory test; CE, crayfish effluent.
Two cohorts of naïve snails (saline, *N*=13; methysergide, *N*=13) were trained in CE with a single 0.5-h training session procedure. Injection was performed 2.5 h before the training and their memory was tested 24 h later.

we hypothesized that the enhanced LTM was caused by an activation of the serotonergic system in our snails following predator detection. Thus, we hypothesized that the increase of 5-HT level in our snails' haemolymph and CNS would also result in a similar effect on memory formation as CE. To test this hypothesis, three cohorts of naïve snails (no-drug group, *N*=10; saline group, *N*=10; 5-HT group, *N*=10) were subject to a single 0.5-h training, but at this time these snails were trained in PW, instead of exposing them to CE. 10.63 μg 5-HT $ml^{-1}$ snail volume (or saline) was injected 3 h before the training, based on our preliminary data. Since, unlike CE, a single 0.5-h training in PW produced ITM lasting for only 3 h, not 24 h, we could use this training procedure to test whether the direct increase of 5-HT level in our snails also resulted in enhanced LTM formation when their memory was tested 24 h later. As seen in Fig. 8, both non-injected and saline-injected groups showed no LTM at 24 h MT (paired *t*-test, *P*>0.05; Fig. 8). However, the 5-HT-injected group was able to produce LTM lasting for 24 h after the single 0.5-h PW training. That is, the number of attempted pneumostome openings in the 24 h MT was significantly reduced compared with those of the TS in 5-HT group (paired *t*-test, *P*<0.01; Fig. 8). Yoked controls confirmed that the decrease in attempted pneumostome openings in the 5-HT group did not resulted from the adverse effect of 5-HT in the 24 h MT (unpaired *t*-test, *P*>0.05; Fig. 8). We therefore conclude that the activation of the serotonergic system is indeed associated with enhancing memory formation in *Lymnaea*.

### Snails in which the osphradial nerve was cut lost their ability to form enhanced LTM in CE

*Lymnaea* modify their behaviour as a result of detecting predator-released kairomones (Dalesman et al., 2006; Orr et al., 2007; Orr and Lukowiak, 2008; Lukowiak et al., 2008). In molluscs, a peripheral sensory structure, the osphradium, processes chemosensory information (Kamardin et al., 2001). The osphradial ganglion, which sits just below the osphradium transfers this chemosensory information to the CNS (Wedemeyer and Schild, 1995). Most interestingly from our perspective, 5-HT neurones have been shown to be present in the osphradium of *Lymnaea* (Nezlin et al., 1994). Osphradial ganglion neurons respond to exogenous serotonin application suggesting that peripheral chemosensory processing is, at least in part, regulated by 5-HT (Kamardin et al., 1999). Thus, we examined whether interrupting information from the osphradium to the CNS would alter the enhancement of LTM formation elicited by CE. Since the CNS receives the input from the osphradium *via* the osphradial nerve, we hypothesized that severing this nerve would disrupt LTM formation. We first

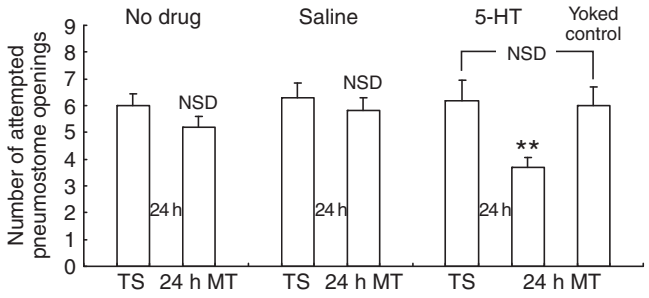


Fig. 8. 5-HT enhanced LTM formation in PW. The no-drug group (*N*=10), saline group (*N*=10) and 5-HT group (*N*=10) received a single 0.5-h training session in PW, which typically produces 3 h ITM. Saline and 5-HT were injected 3 h before the training. When their memory was tested 24 h later, the no-drug and saline groups showed no LTM as we expected (paired *t*-test, both groups *P*>0.05). However, the 5-HT-injected group showed LTM at 24 h although they were trained in PW. That is, the number of attempted pneumostome openings at 24 h was significantly decreased compared with those of the TS (paired *t*-test, *P*<0.01). A yoked control experiment showed that the decreased number of openings in the 24 h MT did not derived from the adverse effect of 5-HT on breathing behaviour in the snails when the TS in the 5-HT group was compared with the 24 h MT in the yoked control group (unpaired *t*-test, *P*>0.05). NSD, no significant difference; **$P$<0.01.

determined whether interrupting the flow of information from the osphradium would impair aerial respiration in the hypoxic condition. Sham (*N*=21) and osphradial-nerve severed (*N*=18) snails were prepared (see Materials and methods), and their basal breathing behaviours compared 3 days later following the surgery. These data showed that the cutting the nerve did not alter the aerial respiratory behaviour. There was no significant difference between the two groups in total breathing time(s) (unpaired *t*-test, *P*>0.05; sham 216.04±31.099 *vs* cut 212.11±26.709), the average pneumostome opening time(s) (unpaired *t*-test, *P*>0.05; sham 9.95±1.51 *vs* cut 10.61±1.29) and the mean number of pneumostome openings (unpaired *t*-test, *P*>0.05; sham 7.47±1.04 *vs* cut 7±0.96).

Next, we determined whether cutting the osphradial nerve would block the enhancing effect CE on LTM formation. Sham (*N*=19) and osphradial nerve cut (*N*=15) snails received a single 0.5 h training in CE and their memory was tested 24 h later. As can be seen (Fig. 9), we found that sham-operated control snails showed LTM formation, whereas the snails with their osphradial nerve severed did not. That is,

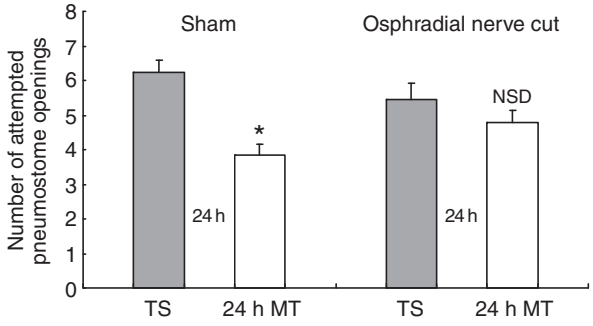


Fig. 9. Severing of the osphradial nerve blocked enhanced LTM formation in CE. Sham-operated snails (*N*=19) and snails in which the osphradial nerve was cut (*N*=15) were trained in CE using a 0.5-h training session. Sham-operated snails still showed enhanced LTM in the 24 h MT as the naive snails did (paired *t*-test, *P*<0.05). However, the snails with the severed nerve did not show enhanced LTM at 24 h (paired *t*-test, *P*>0.05). NSD, no significant difference; **P*<0.05. Grey shading signifies training in CE.

the number of attempted pneumostome openings in the 24-h MT was significantly different than those of the TS in the sham-operated snails (paired *t*-test, $P<0.05$; Fig. 9). However, the number of attempted pneumostome openings in the 24 h MT was not significantly less than those of the TS in the axotomized snails (paired *t*-test, $P>0.05$; Fig. 9).

## DISCUSSION

We tested the hypothesis that the change in the behaviour of the snail caused by predator detection is mediated by a serotonergic system. All of our data presented here are consistent with that hypothesis. Predator detection results in the enhancement of the neuronal and molecular processes that underlie LTM formation. This enhancement takes the form both of: (1) LTM induction with a previously ineffective training procedure (a single 0.5-h session) and (2) significantly longer persistence of LTM (e.g. 5 days *vs* 1 day). In the presence of a serotonin-receptor blocker, the change in the physiological state evoked by CE was not apparent. However, injection of 5-HT into the snail resulted in enhanced LTM formation. Finally, CE appears to be detected by sensory neurones in the osphradium, a peripheral chemosensory organ.

Stressful stimuli alter how events are encoded into memory; sometimes enhancing memory and in others blocking its formation (Shors, 2004). Memory enhancement may not necessarily be a 'good' thing (i.e. think of post traumatic stress disorder) whereas blocking memory formation concerning traumatic events may be a 'good' thing. At present it is unclear what the underlying mechanisms are that determine whether stress will lead to memory enhancement or its blockage. However, it is clear that serotonergic systems have been implicated in a variety of stress-induced responses, such as anxiety, arousal and vigilance (Lowry et al., 2005).

The 'stress' response in molluscs appears to be similar to that in vertebrates. In *Lymnaea* a number of different stressors have been experimentally applied to snails, resulting in alterations to adaptive behaviours (Lukowiak et al., 2008; Lukowiak et al., 2010). For example, snails can be stressed by exposing them to chemical or physical stimuli (e.g. a 25 mmol $l^{-1}$ KCl solution, quinine, garlic solution, alternating hot and cold, repeated scratching of the pneumostome area) all of which can enhance LTM formation (Martens et al., 2007a; Martens et al., 2007b; Lukowiak et al., 2010). More recently a number of ecologically relevant stressors have been shown to alter the snails' physiological state. These include kairomones (predator-derived chemical cues that elicit a response in a prey organism that reduces the probability that the prey will be harmed by the predator) low-levels of environmental calcium, etc (Lukowiak et al., 2008; Orr and Lukowiak, 2008; de Caigny and Lukowiak, 2008; Dalesman and Lukowiak, 2010). In all instances altered behavioural responses are observed indicating that the snail has been affected. Following predator detection the shadow-induced whole-body withdrawal response is more readily evoked, as are the time it takes to emerge from its shell following the whole-body withdrawal response. It is, however, not entirely clear how the altered effect of the stressor is mediated at the neuronal level.

Serotonin plays a major role in the modulation and/or mediation of behavioural arousal regulating motor activities in both invertebrate and vertebrate model systems (Geyer, 1996; Rueter et al., 1997; Weiger, 1997; Gillette, 2006). Predator detection in *Lymnaea* elicits a suite of so-called 'vigilance' behaviours (Rundle and Bronmark, 2001; Orr et al., 2007). Vigilance behaviours are thought to enable an animal to increase its likelihood of avoiding predation (Apfelbach et al., 2005). Here we showed that in the presence of mianserin two of the vigilance behaviours elicited by CE (i.e. increased duration of hiding in their shell and facilitation of the shadow-evoked withdrawal response into their shell) were abolished. Given that serotonin systems play such an important role in mediating behavioural arousal, this result was not too surprising.

Predator detection alters adaptive behaviours beneficial to survival. Here we showed that this enhanced LTM formation in *Lymnaea* is dependent on a serotonergic system. Snails trained in CE show enhanced LTM, but this enhancement is not apparent in the presence of the serotonin-receptor blockers mianserin and methysergide. Moreover, in PW LTM formation was not blocked by mianserin nor did it block intermediate memory formation (ITM). In addition, the direct increase of 5-HT levels in the haemolymph as a result of the injection of exogenous 5-HT prior to a single 0.5-h PW training session led to enhanced LTM. These results are all consistent with the hypothesis that predator detection increases serotonergic activity in *Lymnaea*, which subsequently leads to altered synaptic and/or cellular properties of neurones such as RPeD1, a necessary site for LTM formation, memory reconsolidation, memory extinction and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003d; Sangha et al., 2003b; Sangha et al., 2005). Previously it has been shown that stressors can lead to altered serotonergic tone both in the mammalian brain and CNS of molluscs (Kawahara et al., 1993; Rueter and Jacobs, 1996; Adell et al., 1997; Amat et al., 1998a; Amat et al., 1998b; Marinesco and Carew, 2002; Marinesco et al., 2004a). Furthermore, increasing the serotonergic tone in human subjects is accompanied by an enhanced response to anxiety-related stimuli and enhanced memory consolidation (Harmer et al., 2002; Browning et al., 2007). Our data are consistent with these earlier reports showing stress-related serotonergic modulation of adaptive behaviour.

It is possible that the enhanced LTM seen with training in CE might be the result of prolonged or increased activity of some kinases in RPeD1 following their activation by serotonergic pathways. There are two factors that support this. (1) LTM was not observed following RPeD1 soma ablation. The nucleus of the neuron is no longer present (i.e. no genes) but a functional primary neurite remains that is capable of supporting the synaptic connections necessary to mediate aerial respiratory behaviour and ITM formation (Scheibenstock et al., 2002). (2) Inhibiting protein phosphatase activity or increasing kinase activity causes enhanced LTM formation following a single 0.5-h training session in PW. Moreover, this 'boosting' seen following protein phosphatase inhibition or increased kinase activity was not seen following RPeD1 soma ablation (Rosenegger et al., 2008). Activation of cAMP-dependent protein kinase (PKA) has been shown to be critically involved in initiating transcriptional cascades underlying LTM (Frey et al., 1993; Abel et al., 1997; Huang et al., 2000; Kandel, 2001). Additionally, it was demonstrated that 5-HT induced long-lasting synaptic potentiation in the amygdala of mice, an area implicated in both memory formation and its maintenance following fear conditioning. These changes required both the activation of PKA and gene transcription (Huang and Kandel, 2007). In *Aplysia* temporally distinct patterns of PKA activity (transient *vs* persistent) that are correlated with shorter or longer lasting forms of synaptic plasticity have been shown following different 5-HT application sequences. For example, following a single pulse of 5-HT only short-term synaptic facilitation (STF) is seen; whereas five pulses of 5-HT are required for long-term facilitation (LTF). STF results from a transient PKA activation; whereas, LTF results from a persistent PKA activation (Muller and Carew, 1998). Similar data have been shown in honeybees. For example, a single conditioning trial that typically does not result in LTM, will cause LTM if PKA activity is artificially prolonged (Muller, 2000). More recently, in *Lymnaea* it was shown, in a different learning procedure than the one

used here, that prolonged PKA activation was also necessary for a single-trial reward conditioning leading to LTM (Michel et al., 2008). Thus, it is possible that there is a prolongation of PKA activity in neurones such as RPeD1 as a result of predator detection (i.e. CE) that is mediated by serotonin.

Mianserin application altered the enhanced persistence of LTM in CE following two 0.5-h training sessions with a 1-h interval. In CE the resulting LTM persists for at least 8 days (Orr and Lukowiak, 2008). Here, however, this more persistent LTM was not observed in the mianserin-treated snails. It is worth noting that in the presence of mianserin a 24-h LTM was still observed following the two 0.5 h or the 1 h training session procedures whether of not they were trained in CE or PW. This indicates that the 'basal' processes that underlie the production of LTM following these two procedures were not altered by mianserin. That is, mianserin has no effect on the processes that underlie LTM formation in PW. The fact that mianserin abolished only the longer-lasting LTM (5 days) compared with normal LTM (24 h) in these snails suggests that (1) there is an independent system activated only as a result of the perception of risk (e.g. predator detection), which consequently has a role in the enhanced memory formation through serotonergic modulation, and (2) the activation of the serotonergic system is a necessary component for more persistent LTM formation in CE while this system is not involved in the process of normal memory formation itself.

Non-declarative memory, the form of memory studied here, is stored within the same circuit that mediates learned behaviour (Milner et al., 1998). Thus, the 'memory trace' underlying the LTM studied here is thought to reside within the central pattern generator (CPG) circuit that drives aerial respiratory behaviour. This has been demonstrated in a number of studies (Spencer et al., 1999; Lukowiak et al., 2003) and it has been shown that molecular changes in one of these CPG neurones, RPeD1, are necessary for LTM formation (Scheibenstock et al., 2002). The data obtained here lead us to suspect therefore that respiratory CPG neurons may be subject to modification by a serotonergic system in terms of enhanced and/or more persistent memory formation following predator detection. We know that the activity of RPeD1 is significantly altered as a result of predator detection (Orr et al., 2007); what we do not yet know is whether this alteration of the electrophysiological properties of RPeD1 seen following predator detection is mediated by the serotonergic system. It is known, however, that activation of LPeD1 (a giant, serotonin containing neuron) coincided with inhibition of RPeD1 activity (Tsyganov, 2001). Thus, future studies are needed to determine if predator detection alters LPeD1 activity and if the change in activity is in any way connected with the predator-induced changes observed in RPeD1. Predator-detection-induced changes in adaptive behaviours are reflected in RPeD1 activity, which plays a crucial role in memory formation, reconsolidation, and extinction (Lattal et al., 2006; Parvez et al., 2006; Orr et al., 2007). We will therefore in the future determine whether blocking the serotonergic system in CE prevents the electrophysiological changes in RPeD1 induced by predator detection and ultimately attempt to elucidate the molecular mechanism involved in stress-related memory formation at a neuronal level.

An important finding in the present study is that the enhanced LTM was not observed following the interruption of the information flow (i.e. severing the osphradial nerve) from the osphradium to the CNS. *Lymnaea* detect the predator-released kairomone and as a result make modifications to their behaviour (Dalesman et al., 2006; Orr et al., 2007). Based on the data presented here (Fig. 9) it appears that *Lymnaea* detect CE *via* chemosensory neurones in the osphradium. In molluscs the osphradium is a chemosensory organ perceiving and analyzing various odorants (Wedemeyer and Schild, 1995), and stimulation of the osphradium by various chemical and physical stimuli has been associated with the changes in the responses of CNS neurons (Kamardin et al., 2001). In *Lymnaea* eliciting the activity of the osphradial neurones by current injection was followed by the activity of the CPG neurone RPeD1, suggesting that peripheral information from the osphradium can be relayed to the CNS *via* direct synaptic connection (Bell et al., 2008). Interestingly, immunohistochemical methods have indicated that 5-HT neurons are present in the osphradium of *Lymnaea* (Nezlin et al., 1994). Furthermore serotonin application to the osphradium depolarizes three osphradial ganglionic cells (GC1–3), which synapse on neurons in the central ganglia of *Lymnaea*. It has been suggested (Wedemeyer and Schild, 1995; Kamardin et al., 1999) that this peripheral serotonergic system plays a role in processing chemosensory information. However, since the interaction between central and peripheral nervous system of molluscs, as regards mediation of adaptive behaviours, is complicated and controversial (Lukowiak and Jacklet, 1972; Lukowiak and Colebrook, 1988), further investigation regarding the role of the serotonergic system in respect of chemosensory perception and vigilance behaviours during the exposure to CE is necessary.

In conclusion, our data suggest that enhanced LTM memory formation as well as other anti-predator behaviours as a result of predator detection are mediated or modulated by a serotonergic system. Although changes in adaptive behaviours associated with stressors, such as detection of a predator, cannot be adequately explained by a single neurotransmitter or modulator, our current working hypothesis is that the activation of serotonergic pathways is necessary and sufficient for activating vigilance defensive behaviours and enhanced LTM. The advantages of the *Lymnaea* model system (i.e. a relatively simple nervous system mediating tractable behaviours that exhibit associative learning and LTM) can now be extended to examine how the neural networks associated with the serotonergic system are modulated by ecologically relevant stressors. This model system may in fact serve as a basis to examine mechanisms underlying PTSD or 'panic' attacks in humans.

## ACKNOWLEDGEMENTS

This work was supported by The Canadian Institutes of Health Research.

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