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RESEARCH ARTICLE

A flavonol present in cocoa [(–)epicatechin] enhances snail memory

Lee Fruson, Sarah Dalesman and Ken Lukowiak*

Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada, T2N 4N1

*Author for correspondence (lukowiak@ucalgary.ca)

SUMMARY

Dietary consumption of flavonoids (plant phytochemicals) may improve memory and neuro-cognitive performance, though the mechanism is poorly understood. Previous work has assessed cognitive effects in vertebrates; here we assess the suitability of *Lymnaea stagnalis* as an invertebrate model to elucidate the effects of flavonoids on cognition. (–)Epicatechin (epi) is a flavonoid present in cocoa, green tea and red wine. We studied its effects on basic snail behaviours (aerial respiration and locomotion), long-term memory (LTM) formation and memory extinction of operantly conditioned aerial respiratory behaviour. We found no significant effect of epi exposure (15 mg l^{-1}) on either locomotion or aerial respiration. However, when snails were operantly conditioned in epi for a single 0.5 h training session, which typically results in memory lasting ~3 h, they formed LTM lasting at least 24 h. Snails exposed to epi also showed significantly increased resistance to extinction, consistent with the hypothesis that epi induces a more persistent LTM. Thus training in epi facilitates LTM formation and results in a more persistent and stronger memory. Previous work has indicated that memory-enhancing stressors (predator kairomones and KCl) act *via* sensory input from the osphradium and are dependent on a serotonergic (5-HT) signalling pathway. Here we found that the effects of epi on LTM were independent of osphradial input and 5-HT, demonstrating that an alternative mechanism of memory enhancement exists in *L. stagnalis*. Our data are consistent with the notion that dietary sources of epi can improve cognitive abilities, and that *L. stagnalis* is a suitable model with which to elucidate neuronal mechanisms.

Key words: long-term memory, (–)epicatechin, memory enhancement, *Lymnaea stagnalis*, operant conditioning, flavonol.

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INTRODUCTION

We are who we are because of our abilities to form, maintain and recall memory (Milner et al., 1998; Martin et al., 2000). All aspects of learning, memory and extinction are dynamic and inherently susceptible to modifications *via* environment and lifestyle choices, of which diet is one of the most important. Flavonoids are a group of phytochemicals that are widespread throughout the plant kingdom and whose estimated daily intake among humans is ~1 g day^{-1}, the majority of which is consumed *via* fruits and vegetables (Kühnau, 1976). Phytochemicals have been associated with improvements in both learning and memory in rodents (Matsuoka et al., 1995; Galli et al., 2002; Youdim et al., 2002; Wang et al., 2006). The positive cognitive enhancements have been attributed to the protection and enhancement of neural function and stimulation of neuronal regeneration (Matsuoka et al., 1995; Galli et al., 2002; Youdim et al., 2002; Wang et al., 2006), and also their ability to protect against oxidative stress-induced neuronal death (Inanami et al., 1998). However, *in vitro* studies also indicate that flavonoids may enhance neuronal signalling pathways, but this has yet to be demonstrated *in vivo* (e.g. Spencer, 2010). Therefore, the actions that flavonoids have in improving cognitive function are still unclear.

Lymnaea stagnalis (Linnaeus 1758) will potentially provide an ideal model system in which to investigate the action of flavonols on learning and memory formation. Firstly, this species responds in a highly consistent manner to operant conditioning to reduce aerial respiration, the memory of which is altered by environmentally relevant stimuli, so we can reliably assess how different factors alter memory formation (Lukowiak et al., 1996; Lukowiak et al., 2000; Lukowiak et al., 2010). Secondly, we are able to measure changes at the level of a single neuron [right pedal dorsal 1 (RPeD1)] in the central pattern generator, which controls aerial respiration that coincides with changes in behaviour (Lukowiak et al., 2003; Braun and Lukowiak, 2011). We carry out these measurements using a semi-intact preparation that retains connectivity in the central nervous system (CNS) and with external sensory and motor neurons, ensuring that we are recording 'realistic' activity. Therefore, this species provides an opportunity to assess neuronal signalling properties *in vivo*. However, as the memory-enhancing properties of flavonoids have not previously been shown in invertebrates, we first need to demonstrate that flavonoids can enhance memory formation in *L. stagnalis*.

We can parse memory into a number of different forms, for example, by how long the memory persists following learning. Short-term memory persists for minutes, intermediate-term memory (ITM) persists for a few hours and long-term memory (LTM) persists for days to months to years (Rosenzweig et al., 1993; Lukowiak et al., 2000). Similarly to other species studied (Barondes and Jarvik, 1964; Cohen and Barondes, 1966; Flood et al., 1975; Squire et al., 1975), in our model system, the pond snail *L. stagnalis*, ITM is dependent on the translation of existing RNA transcripts whilst LTM involves the formation of new RNA transcripts and protein synthesis (Sangha et al., 2003d). Extinction is considered a specific type of learning process (Pavlov, 1927; Lattal et al., 2006; Kennedy et al., 2010). During extinction, the behavioural phenotype is similar to 'forgetting'; however, extinction is, in fact, new learning and memory formation that

occludes, but does not erase, previously learned behaviour (Bouton, 1994; Eisenberg et al., 2003). It can be characterised by spontaneous recovery of the trained phenotype with no further stimulus (Pavlov, 1927) or reinstatement upon presentation of the unconditioned stimulus (Myers and Davis, 2002). We have previously demonstrated extinction in *Lymnaea* (McComb et al., 2002), and we further demonstrated that extinction can be extended or enhanced by a number of different means, including blocking newer memory formation (Sangha et al., 2003e; Sangha et al., 2005; Knezevic et al., 2011). Additionally, animals that have apparently forgotten exhibit re-instatement of the original training following a protocol that does not typically result in LTM formation (Sangha et al., 2003b; Carter et al., 2006; Parvez et al., 2006; Kennedy et al., 2010). We chose to assess the effects of (–)epicatechin (epi), as it has been identified as a bioactive molecule *in vivo* (Schroeter et al., 2006) and is also commonly consumed through cocoa, green tea, blueberries and red wine (Spencer, 2008). Epi is capable of crossing the blood–brain barrier in rats following oral ingestion (Abd El Mohsen et al., 2002), and has been shown to improve their spatial memory (van Praag et al., 2007). In *Lymnaea*, water-soluble substances (such as epi) can easily cross the skin membrane, and the snails possess an open circulatory system that allows for direct contact of the bio-active substance with the CNS. With current research favouring a relationship between flavonoids and cognitive enhancement, it is plausible that epi could enhance LTM formation and extend LTM's persistence in the snail. We used *Lymnaea* initially to assess the effect of epi on basic behaviours (locomotion and total breathing time). We then assessed the effect of epi exposure on LTM formation, its persistence and extinction, providing the first test of the effect of flavonoids on invertebrate learning and memory. Finally, we investigated whether the mechanism by which epi alters memory is similar to other environmental stimuli that cause memory enhancement, by assessing the roles of chemosensation *via* the osphradium and serotonergic (5-HT) signalling pathways in LTM enhancement *via* epi in the snail (Il-Han et al., 2010; Karnik et al., 2012a).

MATERIALS AND METHODS

Animals

We used adult *L. stagnalis* (25 mm spire height) from the Dutch laboratory strain, originating from animals collected in the 1950s from canals in a polder located near Utrecht. Animals were reared at the University of Calgary in the Biological Sciences building, where they were maintained in artificial pond water (0.25 g l^{-1} Instant Ocean, Spectrum Brands, Madison, WI, USA) and supplemented with $CaCO_3$ to keep calcium concentrations above 50 mg l^{-1} (Hermann et al., 2009). Approximately 1 week prior to experimentation, the snails were transferred to our laboratory and placed in our standardized oxygenated artificial pond water (0.26 g l^{-1} Instant Ocean plus 80 mg l^{-1} [Ca^{2+}]) (Dalesman and Lukowiak, 2010; Dalesman et al., 2011a). The snails were fed romaine lettuce *ad libitum* and remained at room temperature (20±1°C) on a schedule of 16 h:8 h light:dark at a density of one snail per litre water. Final *N*-values for all experiments are provided in the figure legends.

Drug exposure

Pure epi was obtained from Sigma-Aldrich (St Louis, MO, USA). Based on pilot studies, a solution of 15 mg l^{-1} epi in standard pond water was chosen as it does not alter homeostatic behaviours (L.F., personal observations), and is within what may be consider the equivalent of consumption levels in humans (Kühnau, 1976).

Aerial respiratory behaviour

Lymnaea stagnalis is a bimodal breather. In highly oxygenated conditions it breathes by absorbing oxygen directly across its skin; however, as dissolved oxygen levels drop it switches to aerial respiration using a basic lung opened through a respiratory orifice called the pneumostome (Lukowiak et al., 1996; Lukowiak et al., 2006). Aerial respiration is easily monitored *via* observation of the pneumostome. To investigate whether epi had any deleterious effect on aerial respiration, the total breathing time was measured under hypoxic conditions over a 30 min period both in pond water and epi-pond water.

Snails were placed in 1 litre beakers filled with 500 ml of pond water (PW) or epi-pond water (epi-PW), which had been made hypoxic *via* 20 min of vigorous N_2 bubbling. Bubbling was reduced and continued at a low level during total breathing time recording to maintain the hypoxic environment without disturbing snail activity. Snails were given 10 min to acclimate and then were observed for 30 min while recording breathing behaviour. This gave a total exposure time of 40 min in the epi-treated animals. Each individual was observed in both epi-PW and PW using a randomised block design, where half the snails experienced epi first and half experienced PW first, with a 24 h gap between the two observation periods.

Locomotion

To assess snail locomotion, individual snails were placed into large Petri dishes (14 cm diameter by 2 cm depth) that had been filled with PW (control) or epi-PW to a depth of 1 cm (Dalesman and Lukowiak, 2010). Because snails generally withdrew into their shell upon placement in Petri dish, the observation period did not begin until the snail had re-emerged again, such that the head and tentacles were visible. Distance travelled was then recorded over a period of 15 min using a 2 cm^2 grid that had been etched on the base of the Petri dish. Average velocity over the 15 min session was then calculated and presented as mean crawling rate (mm s^{-1}) (Dalesman and Lukowiak, 2010). Again, a randomised block design was used, with half the snails experiencing epi-PW first and a 24 h gap between locomotion trials.

The single 0.5 h training session procedure

In the Dutch laboratory-reared snails used in these experiments, a 0.5 h training session (TS) typically results in the formation of ITM that persists for ~3 h, but not LTM formation when tested 24 h later (Lukowiak et al., 2000; Sangha et al., 2003d; Orr and Lukowiak, 2008; Braun and Lukowiak, 2011). However, if this training procedure is performed in the presence of certain stressors (e.g. KCl or predator kairomones), LTM results (Martens et al., 2007; Orr and Lukowiak, 2008). Thus if epi caused enhancement of memory formation, we could predict that the single 0.5 h training procedure in the presence of epi would result in LTM at 24 h.

As in the test for total breathing time, 500 ml of PW (control) or epi-PW (treatment) was made hypoxic (≤0.1 ml O_2 l^{-1}) by vigorously bubbling N_2 through the beaker for 20 min. Bubbling was then reduced and continued at a low level during acclimation, training and memory testing to maintain the hypoxic environment without disturbing the animals. Snails were placed into the hypoxic environment for a 10-min acclimation period, followed by a 0.5 h TS, thus giving 40 min total exposure to epi-PW or PW during conditioning. Snails were trained using the standard operant conditioning protocol (Lukowiak et al., 1996), in which the pneumostome is 'poked' with a sharp wooden stick each time the snail attempts to open it. Pokes were forceful enough to cause closure

of the pneumostome but gentle enough so as not cause full withdrawal of the snail into its shell. The number of pokes (i.e. attempted pneumostome openings) was recorded for each individual snail over the 0.5 h TS. To determine whether LTM was formed following the single 0.5 h TS, an identical procedure was performed 24 h later [i.e. a memory test (MT)]. The number of attempted pneumostome openings in the MT was compared with the number in the TS and LTM was considered to be present if the number of attempted openings in the MT was significantly lower than that in the TS (Sangha et al., 2003b; Parvez et al., 2006).

Yoked control

To ascertain that a significant decrease in the number of attempted pneumostome openings seen when snails were trained in epi was due to associative learning and LTM rather than some unknown side effect of the 'drug' or a generalised response to physical stimuli, we performed yoked control experiments. Yoked control snails were subject to the same experimental protocol as above (a single 0.5 h TS followed 24 h later by a 0.5 h MT); however, tactile stimulation during training was not contingent with their pneumostome opening. Instead, each snail in the yoked group was paired with an operantly conditioned snail and was 'poked' in the pneumostome area when the operantly conditioned snail to which it was yoked opened its pneumostome. During MT, the snail is then poked contingent on pneumostome opening. As no memory is formed in PW alone, yoked control sessions were performed in epi-PW only.

The two 0.5 h TS procedure

To further investigate the effects of epi-PW on LTM formation and its persistence, we subjected snails to a procedure consisting of two 0.5 h TSs (TS1 and TS2) separated by a 1 h interval, using identical methods to those outlined above for a single TS. This procedure results in LTM formation that typically persists for 24 h, but not 72 h in PW alone (Lukowiak et al., 2000; Sangha et al., 2003d; Parvez et al., 2005; Braun and Lukowiak, 2011; Dalesman and Lukowiak, 2011). Therefore, we tested LTM following training in either PW or epi-PW either 24 or 72 h following the two 0.5 h TSs. LTM memory following TS2 was considered present if the number of attempted pneumostome openings during the MT was significantly less than that during TS1, but not significantly greater than that during TS2 (Lukowiak et al., 1996; Lukowiak et al., 2000).

Extinction training following the two 0.5 h TS procedure

We further investigated the possible enhancing effects of epi on LTM formation by examining whether snails trained in epi-PW were more resistant to extinction. The learned association between pneumostome opening and poking was extinguished by placing snails in the same hypoxic environment as that of associative training, but instead of poking the snails in the pneumostome they were allowed to freely perform aerial respiration. This protocol results in the occlusion of the previously formed memory (i.e. that aerial respiration is associated with a negative stimulus, the 'poke') with a new memory (i.e. that aerial respiration does not result in negative consequences). The extinction protocol used here involved three 0.5 h extinction (Ext) sessions (McComb et al., 2002; Sangha et al., 2003a; Sangha et al., 2003c).

In the extinction experiments, snails were first subjected to an observation session (Obs) 24 h prior to training, in which they were placed in hypoxic PW or hypoxic epi-PW for 0.5 h (TS1 and TS2, respectively). During this time, the number of pneumostome openings was calculated for each snail in the absence of tactile stimulation (i.e. openings measured here cannot be directly compared with those during TS or MT). Snails were then trained 24 h later using two 0.5 h TSs as outlined above. One hour following either the 24 or 72 h MT, the snails were then subjected to three 0.5 h extinction sessions (Ext1–3) using the same procedure as during Obs (see Figs 5, 6 for the experimental timeline). Ext1 was administered 1 h after MT and was followed 1 h later by Ext2. The final extinction session (Ext3) was administered 24 h after Ext2.

Snails were considered to have resisted extinction (retained LTM) if the number pneumostome openings during the extinction session (i.e. in the absence of physical stimuli) was significantly less than that during the Obs session.

Osphradial input and epi-PW training

Input from the osphradium has been found to modulate the memory-enhancing response of both KCl and predator kairomones (Il-Han et al., 2010; Dalesman et al., 2011b; Karnik et al., 2012a), but is not necessary for standard memory formation in control conditions (Il-Han et al., 2010; Dalesman et al., 2011b) or normal aerial breathing behaviour (Karnik et al., 2012b). Therefore, we wanted to test whether osphradial input is necessary for the memory-enhancing effects of epi.

Osphradial input was prevented by severing the osphradial nerve connecting this organ to the CNS, and the response of *L. stagnalis* to training in epi was compared with that of a sham-operated control group also trained in epi. Operative procedures were carried out 3 days prior to experiments to allow sufficient time for the recovery of normal breathing behaviour (Karnik et al., 2012b). Snails were first anaesthetised using iced pond water, and were subsequently injected with 2 ml of 50 mmol l^{-1} $MgCl_2$ *via* the foot into the haemocoel, which prevents withdrawal into the shell. The animals were then placed into a dissection dish and a small slit was made in the skin adjacent to the osphradium to access the nerves. In the sham animals, the small slit was made, but the nerve was left intact. In the osphradially cut animals, the nerve was severed proximal to the osphradium. The animals recovered rapidly from both procedures and no further action was required to close the incision. Training for both surgery groups was carried out in epi-PW 3 days following surgery following a protocol identical to the single 0.5 h TS procedure above, and snails were tested for memory 24 h later.

Blocking serotonergic signalling and epi-PW training

Serotonergic signalling is thought to mediate arousal responses caused by noxious stimuli, e.g. predator detection, in several molluscan models (Katz and Frost, 1995; Jing and Gillette, 2000; Marinesco and Carew, 2002; Marinesco et al., 2004). Additionally, it is necessary for the enhancement of memory formation by the presence of predator kairomones during operant conditioning in *Lymnaea* (Il-Han et al., 2010). Therefore, whether or not osphradial sensory input is required, we considered that memory enhancement may still be modulated *via* a serotonergic signalling system.

To prevent serotonergic signalling, we used the serotonin-receptor antagonist mianserin (Sigma-Aldrich), which has been used to successfully block serotonin-modulated memory enhancement without other side effects on basic behaviour and memory formation under control conditions in *Lymnaea* (Il-Han et al., 2010). Mianserin (7.5 mg l^{-1}) was 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), and 100 µl of the solution was injected into the haemocoel through the foot of the snails 2.5 h prior to training. As a control for the injection

procedure, we also injected a control group with the same volume of *Lymnaea* saline only. Training for both saline-only and mianserin-exposed groups was carried out in epi-PW following a protocol identical to that of the single 0.5 h TS procedure above, testing for LTM at 24 h.

Statistical analysis

All data were analysed using repeated-measures ANOVA (RM-ANOVA) in SPSS 17.0 (IBM, Armonk, NY, USA), which allowed for within-subject comparisons over time, as well as between-subject comparisons for the effects of treatment. Mauchley's test for sphericity was used to assess homogeneity of variance, and the more conservative Greenhouse–Geisser *P*-values were used where assumptions of homogeneity of variance were not met. Where significant within-subject differences were identified, *post hoc* paired *t*-tests were used to compare the response of naive snails, either during the initial training session or breathing rate pre-training, with their subsequent behaviour. Where significant between-subject differences were found during operant conditioning, Tukey's pair-wise comparisons were used to assess where these differences lay, particularly as it could be differences in the number of initial stimuli during training that resulted in differences in memory formation.

Total breathing time and crawling rates were analysed using the order in which snails were exposed to control conditions or epi-PW as the within-subject factor and the response to treatment (control *versus* epi) as the between-subject factor.

The response to a single 0.5 h TS was analysed using treatment group (epi *versus* control) as the between-subject factor and response to training (TS *versus* MT) as the within-subject factor. Whether the response to training in epi was due to learning not to open the pneumostome in response to a contingent stimulus or a general response to a repeated physical stimulus in hypoxia was analysed using training regime (trained *versus* yoked control) as the between-subject factor and the response to training (TS *versus* MT) as the within-subject factor.

The response to two 0.5 h TSs was analysed separately for snails tested 24 or 72 h following training. Data were analysed using the exposure treatment (control *versus* epi) as the between-subject factor and the response to training (TS1 *versus* TS2 *versus* MT) as the within-subject factor.

Breathing behaviour in hypoxia in the absence of a physical stimulus prior to (Obs) and following (Ext1–3) training was analysed separately for groups tested for memory 24 or 72 h following two 0.5 h TSs. Data were analysed using exposure treatment (control *versus* epi) as the between-subject factor and time at which breathing was assessed (pre-training Obs *versus* Ext1 *versus* Ext2 *versus* Ext3) as the within-subject factor.

Assessment of the effect of severing the osphradial nerve on memory formation in the presence of epi-PW was analysed using surgical procedure (osphradial cut *versus* sham) as the between-subject factor and the response to training (TS *versus* MT) as the within-subject factor.

The effect of mianserin on memory formation in the presence of epi-PW was assessed using injection treatment (mianserin + saline vehicle *versus* saline vehicle alone) as the between-subject factor and the response to training (TS *versus* MT) as the within-subject factor.

RESULTS

Aerial respiratory behaviour

Exposure to epi did not alter total breathing time ($F_{1,18}$=0.23, P=0.636; Fig. 1) and there was also no effect of the order in which snails were tested in control or epi exposure conditions ($F_{1,18}$=1.41, P=0.251) or any interaction between order in which exposures were received and the response to epi ($F_{1,18}$=0.48, P=0.499). We concluded that epi does not alter breathing rate in the absence of a physical stimulus.

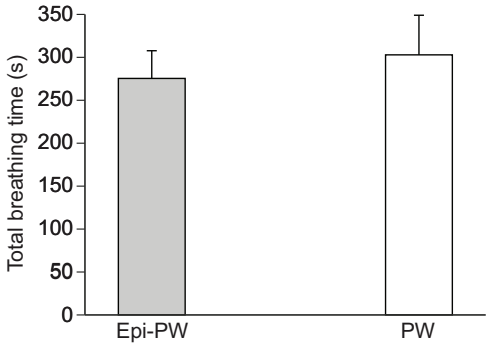

Fig. 1. Aerial respiratory behaviour in *Lymnaea stagnalis* is not significantly altered by epicatechin. The total breathing time (s) is plotted for snails in epicatechin-pond water (epi-PW; grey) and pond water (PW; white). There was no statistical difference in the time spent performing aerial respiration between the two conditions, irrespective of whether they were examined first in pond water or epicatechin-pond water (15 mg l^{-1}). N=20.

Locomotion

Exposure to epi did not alter crawling rate ($F_{1,40}$=0.24, P=0.630; Fig. 2); there was also no effect of the order in which snails were tested in control or epi exposure conditions ($F_{1,40}$=1.47, P=0.233) or of the interaction between order of treatment and the response to epi ($F_{1,40}$=2.86, P=0.098). Therefore, locomotory behaviour was not altered by the presence of epi.

Single 0.5 h operant conditioning

The response to training was dependent on the presence of epi (RM-ANOVA, interaction between treatment and response to training: $F_{1,43}$=6.39, P=0.015; Fig. 3). Snails given a single 0.5 h TS in PW did not form LTM (t=0.35, P=0.728, N=24), whereas those trained in epi-PW exhibited LTM (t=5.11, P<0.001, N=21). There was also a significant overall effect of the response to training (RM-ANOVA, main effect of training: $F_{1,43}$=9.56, P=0.003) and a marginally non-significant overall effect of treatment (RM-ANOVA, main effect of treatment: $F_{1,43}$=4.07, P=0.0501). The number of attempted breaths did not differ between the two treatment groups during TS (Tukey's test: P>0.05), but was significantly lower in the epi-PW group during MT (Tukey's test: P<0.05). This indicates that a difference in response to training was not caused by differences in the number of stimuli epi *versus* control animals received during TS, but a difference in their recall of training 24 h later. This is consistent with the hypothesis that epi enhances LTM formation.

Yoked control

We found that the significant decrease in the number of attempted pneumostome openings in the memory test session (MT or MT-yoked) was dependent on whether snails received contingent tactile stimulation to the pneumostome opening or the non-contingent 'yoked' tactile stimuli (RM-ANOVA, interaction between training regime and response to training: $F_{1,26}$=9.82, P=0.004; Fig. 4). Where the presentation of the tactile stimulus was contingent with attempted pneumostome opening, there was a significant decrease in the number of attempted openings between TS and MT (t=4.38,

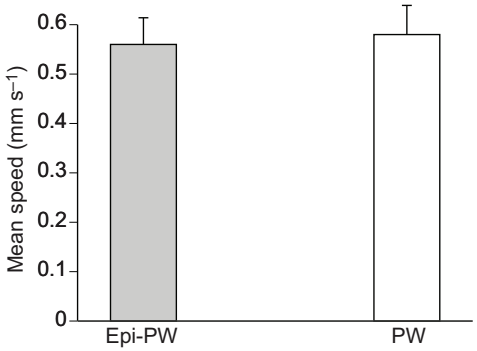

Fig. 2. Locomotory behaviour in *L. stagnalis* is not significantly altered by epicatechin. The mean speed (mm s^{-1}) of snails is plotted in both epicatechin-pond water (epi-PW; grey) and pond water (PW; white). There was no statistical difference in speed of locomotion between the two conditions, irrespective of whether they were examined first in pond water or epicatechin-pond water (15 mg l^{-1}). *N*=42.

P=0.001, *N*=10); however, in the yoked control snails there was no significant decline in the number of attempted pneumostome openings between TS and MT-yoked (*t*=–1.03, *P*=0.320, *N*=10). Overall, there was a significant effect of training regime (RM-ANOVA, main effect of training regime: $F_{1,26}$=10.85, *P*=0.003) but no significant main effect of the response to training (RM-ANOVA, main effect of response to training: $F_{1,26}$=1.84, *P*=0.187). As yoked and contingently trained animals receive an identical number of stimuli during training, these data show that operant conditioning of epi-treated snails results in LTM *via* the association of pneumostome opening with tactile stimulation of the pneumostome rather than a generalised response to the stimulus or repeated epi-PW exposure.

Two 0.5 h TSs followed by MT at 24 h and extinction

There was no significant effect of exposure treatment (i.e. PW *versus* epi-PW) on the number of attempted pneumostome openings during TS1 and TS2 or the MT (RM-ANOVA, main effect of epi treatment: $F_{1,18}$=2.68, *P*=0.119; interaction between the response to training and epi treatment: $F_{2,36}$=0.08, *P*=0.926; Fig. 5). Both groups demonstrated a response to training (RM-ANOVA, main effect of training: $F_{2,36}$=18.60, *P*<0.001; Fig. 5) by significantly reducing the number of attempted pneumostome openings relative to TS1 during TS2 (control: *t*=3.74, *P*=0.004, *N*=11; epi: *t*=3.00, *P*=0.017, *N*=9) and MT (control: *t*=3.89, *P*=0.003, *N*=11; epi: *t*=3.80, *P*=0.005, *N*=9). We found no significant difference between control PW and epi-PW snails in the number of breathing attempts during training sessions or the test session (Tukey's test: *P*>0.05 for all comparisons). That is, associative learning and LTM were present in both PW and epi-PW groups when tested at 24 h.

The number of pneumostome openings in the Obs and extinction sessions was dependent on both time following the memory test and also whether snails were trained in control PW conditions or epi-PW (RM-ANOVA, interaction between time and exposure treatment: $F_{3,54}$=3.10, *P*=0.034; Fig. 5). In the control PW group, the number of pneumostome openings was significantly less during Ext1 than during Obs (Ext1: *t*=4.92, *P*=0.001, *N*=11), but during Ext2 and Ext3, the number of breaths did not significantly differ from Obs (Ext2: *t*=1.41, *P*=0.188, *N*=11; Ext3: *t*=–0.09, *P*=0.927, *N*=11; Fig. 5A). That is, memory extinction occurred during Ext2 and Ext3. However, in the epi-PW group, the number of

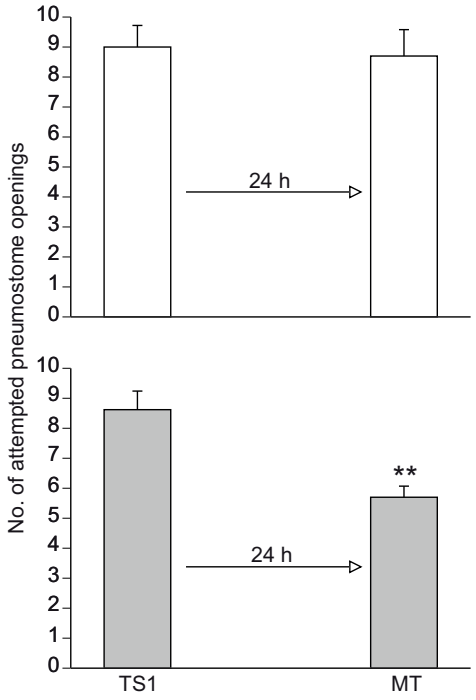

Fig. 3. Epicatechin (15 mg l^{-1}) enhances long-term memory (LTM) formation in *L. stagnalis*. Snails received a single 0.5 h training session (TS1) in either pond water (top; white bars) or epicatechin-pond water (bottom; grey bars) and were tested for LTM 24 h later (MT). Training snails in epicatechin-pond water resulted in a LTM when tested 24 h after TS1. However, snails trained in a similar manner in pond water did not form LTM. **Significant difference between TS1 and MT (*P*<0.01).

pneumostome openings was significantly less than the number in the Obs session in all three extinction sessions (Ext1: *t*=3.62, *P*=0.007, *N*=9; Ext2: *t*=3.65, *P*=0.006, *N*=9; Ext3: *t*=2.80, *P*=0.023, *N*=9; Fig. 5B). Therefore, in the snails trained in epi-PW, extinction did not occur.

We concluded that although both groups exhibit LTM 24 h after training, the memory formed in the epi-PW group was stronger based on it being more resistant to extinction.

Two 0.5 h TSs followed by MT at 72 h and extinction

If we tested both control PW and epi-PW snails 72 h after TS2 there was a significant effect of exposure treatment on the response to training (RM-ANOVA, interaction between response to training and exposure: $F_{2,44}$=6.21, *P*=0.004; Fig. 6). In the control PW group, snails demonstrated learning (TS1 *versus* TS2: *t*=4.52, *P*=0.001, *N*=12), but no LTM at 72 h (TS1 *versus* MT: *t*=0.44, *P*=0.67, *N*=12; Fig. 6A). In the epi-PW group, however, snails demonstrated learning as seen in the control group (TS1 *versus* TS2: *t*=3.63, *P*=0.004, *N*=12) but also demonstrated LTM 72 h following TS2 (TS1 *versus* MT: *t*=3.89, *P*=0.003, *N*=12; Fig. 6B). There was also a significant effect of training (RM-ANOVA, main effect of training: $F_{2,44}$=15.58, *P*<0.001) and overall effect of treatment (RM-ANOVA, main effect of treatment: $F_{1,22}$=10.38, *P*=0.004). We found no significant difference between control PW and epi-PW-treated snails in attempted breaths during the training sessions (Tukey's test: *P*>0.05); however, the number of attempted breaths 72 h following training was significantly higher in the control group compared with the epi-treated group (Tukey's test: *P*<0.05). Together, these data are consistent with the hypothesis that epi causes an enhancement of LTM formation.

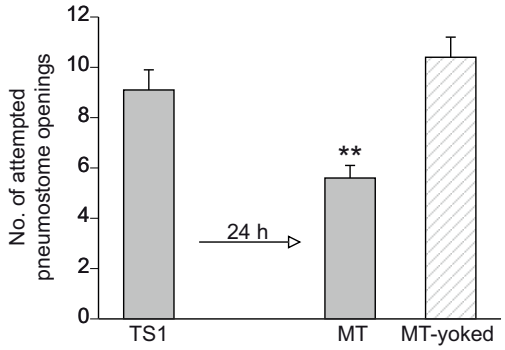

Fig. 4. Yoked control snails do not exhibit LTM but operantly conditioned snails do when trained in epicatechin-pond water. All snails received a single 0.5 h training session. In the operant conditioning group, the tactile stimulus was delivered as the snail attempted to open its pneumostome (i.e. contingent stimulation). However, in the yoked control group, snails received the tactile stimulus when the snail they were yoked to attempted to open its pneumostome. Thus, the yoked control snails received non-contingent stimulation. In the memory test session 24 h later (MT), both groups received contingent stimulation. Yoked control snails (MT-yoked; striped bar) did not exhibit LTM whereas the operantly conditioned group (MT; grey bar) did. **Significant difference between TS1 and MT (P<0.01).

We next tested whether snails trained in epi that possessed LTM at 72 h following TS were also more resistant to extinction than control snails, which did not exhibit LTM. In analyzing the number of pneumostome openings in the extinction sessions and the Obs session, there was no significant effect of treatment group on breathing rate (RM-ANOVA, main effect of treatment: $F_{1,22}$=2.85, P=0.105; interaction between treatment and time: $F_{2.32,51.12}$=1.77, P=0.175); however, the number pneumostome openings taken changed significantly over time between pre-test and the extinction sessions (RM-ANOVA, main effect of time: $F_{2.32,51.12}$=12.143, P<0.001; Fig. 6). In the control PW group, the number of pneumostome openings during Ext1 was significantly lower than pre-test (t=3.08, P=0.010, N=12), but neither Ext2 (t=0.81, P=0.436, N=12) nor Ext3 (t=0.04, P=0.965, N=12) differed significantly from pre-training (Fig. 6A). In the epi-PW group (Fig. 6B), snails in both Ext1 (t=6.01, P<0.001, N=12) and Ext2 (t=4.80, P=0.001, N=12) had significantly fewer pneumostome openings than during the pre-test, but not during Ext3 (t=1.78, P=0.103, N=12), consistent with the hypothesis that training in epi-PW results in a memory that is more resistant to extinction.

We concluded that epi both enhanced LTM formation, increasing retention duration following two 0.5 h TSs, and increased resistance to extinction.

Severing input from the osphradium

Preventing input from the osphradium to the CNS by severing the osphradial nerve did not alter the effect of epi on LTM formation (interaction between surgery and response to training: $F_{1,30}$=0.08, P=0.787; Fig. 7). Snails in both surgery groups formed LTM at 24 h following a single 0.5 h TS (main effect of training: $F_{1,30}$=16.03, P<0.001), as both groups showed a significant decline in pneumostome opening attempts between TS and MT (sham: t=3.97, P=0.001, N=16; cut: t=2.42, P=0.029, N=16). Additionally, there was no overall effect of surgical procedure on LTM formation (main effect of surgery: $F_{1,30}$=1.49, P=0.232). Initial breathing attempts during TS or those in trained animals during MT did not differ between surgical groups (Tukey's test: P>0.05 for both comparisons). Therefore, osphradial input does not affect breathing attempts during training and testing, and is also not necessary for the memory enhancing effects of epi-PW.

Blocking 5-HT

Blocking serotonergic signalling pathways using mianserin did not alter the effect of epi-PW on LTM formation (interaction between surgery and response to training: $F_{1,52}$=1.22, P=0.275; Fig. 7). Snails formed LTM at 24 h following a single 0.5 h TS irrespective of whether they received a mianserin + saline injection or saline only (main effect of training: $F_{1,52}$=20.33, P<0.001), as both groups showed a significant decline in pneumostome opening attempts between TS and MT (saline control: t=4.25, P=0.001, N=28; mianserin: t=2.25, P=0.033, N=26). There was no overall effect of injection protocol on LTM formation (main effect of injection treatment group: $F_{1,52}$=0.52, P=0.474). Further, comparisons between saline- and mianserin-injected snails confirm that these groups do not differ in pneumostome opening attempts during either TS or MT (Tukey's test: P>0.05 for both comparisons). We conclude that serotonergic signalling pathways are not required for the memory-enhancing effects of epi-PW.

DISCUSSION

Our data presented here provide the first support for flavonoid-modulated enhancement of cognitive function in an invertebrate. *Lymnaea stagnalis* responds to a number of bio-active compounds that have the capacity to alter memory formation (Rosenegger et al., 2004; Lukowiak et al., 2008; Lukowiak et al., 2010). The effects of these compounds often mirror those seen in vertebrates, strengthening the case for *Lymnaea* as a model to study the mechanism of effect. As flavonoids have been found to enhance cognitive ability in mammals (Youdim et al., 2002; Wang et al., 2006; Spencer, 2008), we hypothesized that training snails in epi, a flavonoid component of, among other foods, cocoa, would enhance LTM formation in the pond snail. We demonstrated this in *L. stagnalis* in a number of different ways: (1) training snails with a single 0.5 h TS in epi-PW results in LTM, whereas in control snails LTM is not present; (2) when a training procedure that typically results in LTM only persisting 24 h, training in epi results in LTM that persists for at least 72 h; and (3) epi exposure during training and extinction sessions rendered snails more resistant to extinction, consistent with the notion that epi enhanced LTM not only in terms of persistence (i.e. lasted longer) but also in terms of strength (i.e. more resistant to extinction). The enhanced ability of snails trained in epi to form a more persistent and stronger LTM was not due to a 'side effect' of the drug on overall snail behaviour, as there were no changes to important behavioural traits. Exposure of snails to 15 mg l^{-1} of epi-PW did not alter locomotion, aerial respiration or the number of pneumostome opening attempts during the initial training sessions relative to snails in control PW. Additionally, yoked control snails trained in epi-PW do not exhibit LTM; therefore, the decrease in breathing attempts in trained snails was not due to prolonged effects of drug exposure or a generalised sensitization to a physical stimulus. Enhancement of LTM following exposure to methamphetamine has also been shown to occur at a concentration that did not alter homeostatic aerial respiratory behaviour (Kennedy et al., 2010). Thus, we strongly conclude that epi directly affects the activity of neurons that are necessary for LTM formation.

Previous work with *L. stagnalis* has shown memory enhancement in response to a variety of different stimuli, including the bio-active compounds mentioned above (Carter et al., 2006; Kennedy et al.,

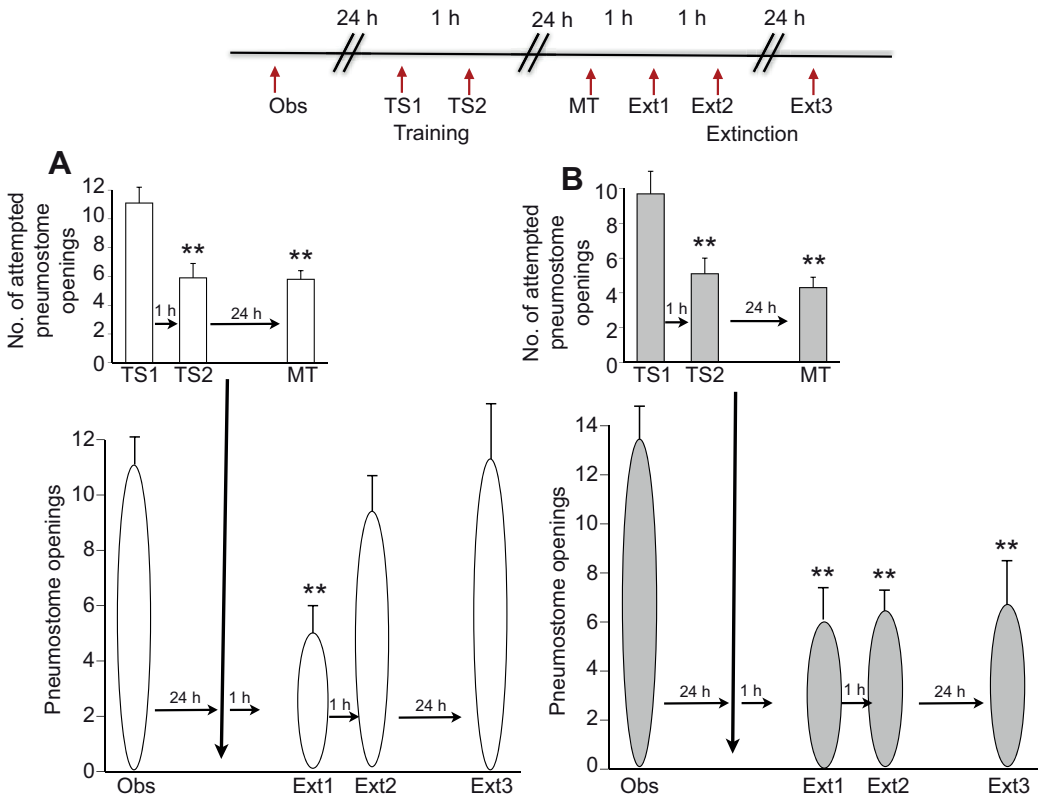

Fig. 5. Training in epicatechin-pond water results in LTM and a memory more resistant to extinction in *L. stagnalis*. On the top portion of the figure, a time line of the experiment is presented. Snails were either operantly conditioned in pond water (A; white bars) or epicatechin-pond water (B; grey bars). Twenty-four hours before training, both groups of snails were placed in hypoxic pond water or hypoxic epicatechin-pond water and the number of pneumostome openings of each snail were tabulated (Obs). Both groups 24 h later then received identical training in either pond water or epicatechin-pond water consisting of two 0.5 h training sessions separated by 1 h (TS1 and TS2) followed by a memory test 24 h later (MT). Both groups exhibited LTM. One hour following the MT (righthand portion of figure), snails were again placed in hypoxic pond water or hypoxic epicatechin-pond water and the number of pneumostome openings of each snail was tabulated. This was the first extinction session (Ext1). All snails then 1 h later received a second extinction session (Ext2) and finally 24 h later they received the third extinction session (Ext3). Extinction occurred in the snails trained in pond water but not in snails trained in epicatechin-pond water, as the number of pneumostome openings in the epi group in all extinction sessions was significantly less than in the pre-test session. **Significant difference in pneumostome openings during Ext1 or 2 or 3 compared with Obs, and between TS2 or MT and TS1 (P<0.01).

2010), KCl, quinine-HCl, rapid cooling or warming (Martens et al., 2007) and predator kairomones (Orr and Lukowiak, 2008). Recently, we have started to elucidate the mechanisms by which these different stressors enhance LTM formation. One ‘target’ we investigated is input from the osphradium, a chemosensory organ that responds to a wide variety of chemical stimuli (Wedemeyer and Schild, 1995; Kamardin et al., 1999; Kamardin et al., 2001). Thus far we have only assessed the response to two of these stressors, KCl and predator kairomones. Our work shows that both of these stressors are sensed *via* the osphradium, causing an increase in the afferent activity of the osphradial nerve connecting this organ to the CNS (Il-Han et al., 2010; Karnik et al., 2012a). This input alters activity in RPeD1, a neuron in the central pattern generator that controls aerial respiration and whose presence is necessary for LTM (Scheibenstock et al., 2002), reducing excitability following external predator kairomone or KCl exposure, thus ‘priming’ the neuron for LTM formation (Karnik et al., 2012a). Additionally, the response to predator kairomones is also dependent on a 5-HT signalling pathway (Il-Han et al., 2010), which we considered may be the mechanism by which all memory enhancement occurs in *Lymnaea*.

Contrary to our hypothesis implicating 5-HT as the primary modulator of memory enhancement, we found that blocking the serotonergic signalling pathway using mianserin did not alter enhanced LTM formation in the presence of epi. Additionally, the effects of epi were not dependent on input from the osphradium. We have previously found that stressors that block memory formation in the snail – low calcium availability, crowding and heavy metal exposure – are modulated *via* different sensory systems (Dalesman et al., 2011b; Byzitter et al., 2012), so in itself the lack of osphradial involvement is not surprising. However, this result does raise two interesting questions. Firstly, is there is a ‘global’ mechanism by which memory is enhanced in the CNS in *Lymnaea*, i.e. is the serotonergic signalling pathway only necessary for information transmission *via* the osphradial nerve and does an alternate signalling system modulate memory enhancement? Alternatively, there may be more than one signalling pathway in the CNS that can lead to memory enhancement. Secondly, and perhaps most relevant for the use of *Lymnaea* as a model to investigate the cellular effects of flavonoids in the brain, is the effect of epi due to direct effects of this compound in the CNS or due to an alternative external sensory system? Using a semi-intact preparation, where the CNS is separated from the body of the snail, allowing sensory systems and the CNS to be exposed in isolation whilst maintaining connectivity (Karnik et al., 2012a), will allow us to address this second question in future work.

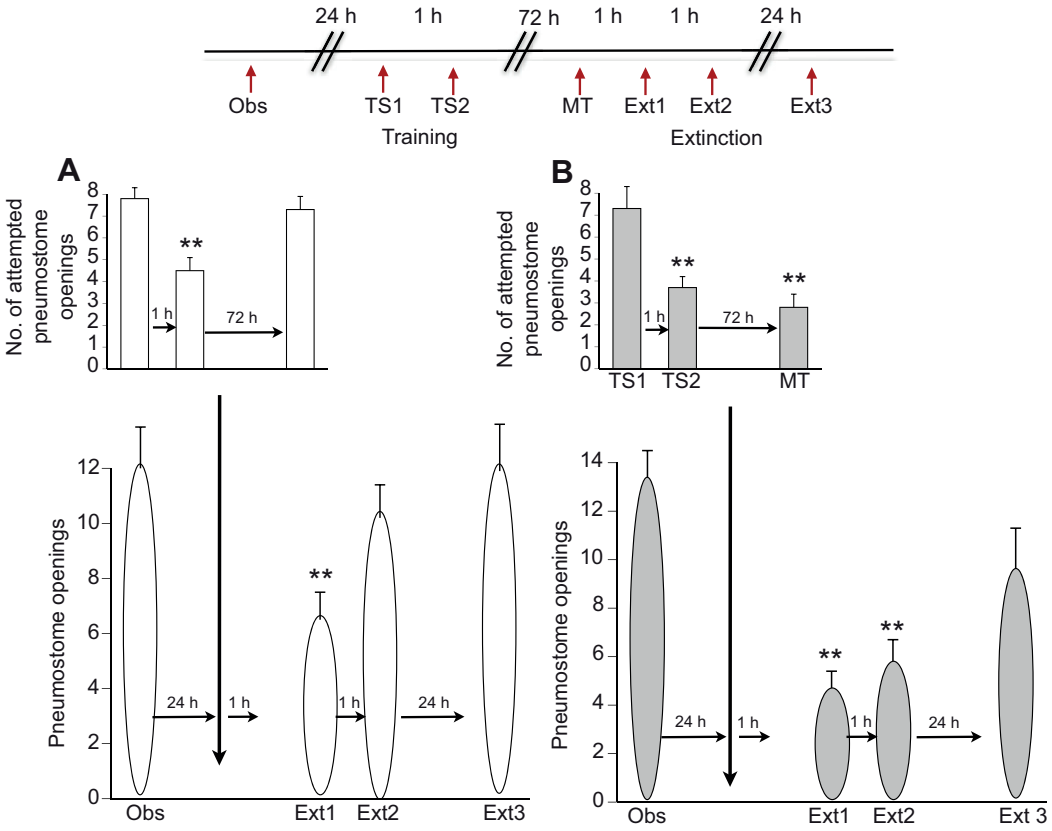

Fig. 6. Training in epicatechin-pond water results in LTM and a memory more resistant to extinction in *L. stagnalis*. As in Fig. 5, except LTM was tested 72 h after TS2. Snails trained in epicatechin-pond water (grey bars) exhibited LTM 72 h after TS2 whereas snails trained in pond water (white bars) did not. Extinction training occurred as in Fig. 5. Notice here that snails trained and extinguished in epicatechin-pond water showed more resistance to extinction than those trained in pond water. **Significant difference in pneumostome openings during Ext1 or 2 or 3 compared with Obs, and between TS2 or MT and TS1 (P<0.01).

There is no evidence in the literature defining a specific mechanism by which epi enhances memory. However, there have been many studies in mammals suggesting various ways in which dietary flavonoids may exert such beneficial effects on the CNS. One hypothesis is that flavonoids act as antioxidants, protecting neurons from injury caused by oxidative stress (Rice-Evans et al., 1996). Oxidation of a wide range of chemicals within the body and the production of radicals results in oxidative damage at the cellular level. This oxidative damage can occur in the brain, resulting in the senescence of neurons (Hanasaki et al., 1994). Antioxidants alleviate this damage by neutralizing reactive oxygen; however, the extent to which flavonoids act as antioxidants is highly variable, as their ability to scavenge and neutralize reactive oxygen species (hydroxyl radicals and superoxide anions) fluctuates from being very effective to having no effect at all (Hanasaki et al., 1994). Watson et al. (Watson et al., 2012) recently demonstrated that an antioxidant (α-tocopherol) can reverse reductions in neuron excitability, which correlate with reductions in memory-forming capabilities induced by chemical oxidant stress or the effects of aging. In a wash directly over the CNS in *Lymnaea*, antioxidant effects were evident within 30 to 40 min, which is within the time scale of exposure in our study, assuming rapid absorption across the skin. Whilst Watson et al. did not assess whether memory could be enhanced in young unstressed animals, these data supports the positive effects of antioxidants on memory formation in *Lymnaea*.

In addition to antioxidant properties, cocoa-derived flavonoids have been shown to increase blood flow to the CNS (Dinges, 2006; Fisher et al., 2006), which has been linked to neurogenesis (Gage, 2000) and improvements in memory (Palmer et al., 2000). However, this is unlikely to account for improved memory in *Lymnaea* as it possess an open circulatory system. Alternatively, another more 'likely' mechanism *via* which long-term memory may be altered by epi is indicated by the effect of flavonoids on long-term potentiation (LTP). LTP is widely considered to be a cellular mechanism underlying certain forms of memory (Bliss and Collingridge, 1993) and is thus a likely candidate for LTM enhancement by epi. How substances such as epi or other flavonoids alter LTP are unknown, although studies suggest that flavonoids may modulate kinase activity [e.g. mitogen-activated protein kinase (MAPK) cascade] and signalling cascades lying downstream of these kinases (Spencer, 2007). The protein kinase/MAPK signalling cascades have been shown to be necessary for both intermediate and long-term memory formation in *Lymnaea* (Rosenegger and Lukowiak, 2010). Therefore, alteration of protein kinase activity by epi may potentially be the mechanism by which memory formation is enhanced in *Lymnaea*. Our future work will aim to test the alternate theories of memory enhancement by epi using the snail as a model system.

In addition to enhancing the ability of *Lymnaea*, our results also show that epi exposure diminished the rate of extinction. Following training and testing 24 h post-training, the memory in epi-exposed

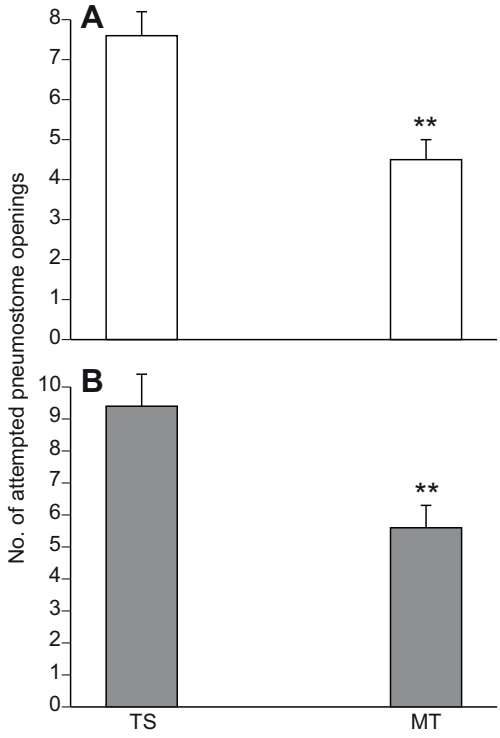

Fig. 7. Preventing osphradial input does not block memory enhancements by epicatechin in *L. stagnalis*. Three days following either sham surgery (A) or surgery to sever the osphradial nerve (B), snails received a single 0.5 h training session in epicatechin-pond water. Both sham-operated animals and those that had their osphradial nerve severed exhibited memory 24 h following a single training session in epicatechin-PW. **Significant difference between TS1 and MT (P<0.01).

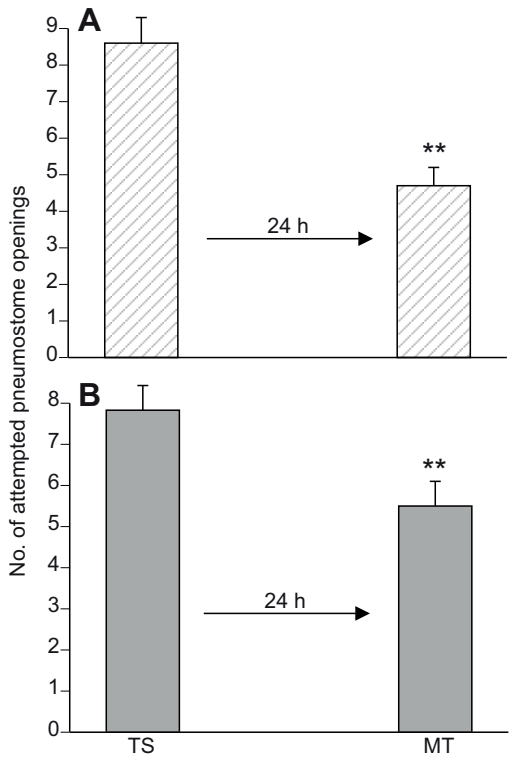

Fig. 8. Blocking serotonergic signalling pathways does not prevent memory enhancement by epicatechin in *L. stagnalis*. Two and a half hours following injection of either saline alone (A) or saline vehicle + mianserin (B), snails received a single 0.5 h training session in epicatechin-pond water. Both saline-injected animals and those that had received mianserin treatment exhibited memory 24 h following a single training session in epicatechin-PW. **Significant difference between TS1 and MT (P<0.01).

snails showed no extinction compared with control animals, which demonstrated breathing rates similar to those of to naive animals following a single extinction session. We saw a very similar pattern when snails were trained and tested for memory at 72 h: control animals only showed a reduction in breathing rate during the first extinction session, whereas epi-exposed animals only demonstrated extinction during the third session. In the extinction procedure we employed here, the first extinction session is presented to the snails 2 h after the MT. In the MT session, snails receive contingent stimulation to their pneumostome as they attempt to open it and this will result in activation of a memory trace. This explains, in part, why the snails in the control PW group exhibited memory in Ext1, even 72 h following TS2. The residual memory trace has been found in previous work (Parvez et al., 2005; Parvez et al., 2006; Braun and Lukowiak, 2011; Dalesman and Lukowiak, 2012), and would be present and activated by the tactile stimuli delivered to the snail during the MT. However, as seen in the control snails, extinction occurs rapidly thereafter. In the epi-treated snails, however, in both the 24 and 72 h procedures, resistance to extinction was shown. Thus, we feel confident that epi produces not only a more persistent LTM, but also a stronger LTM.

This study in *L. stagnalis* has shown that the dietary flavonoid epi has the ability to enhance LTM formation, producing phenotypic effects similar to those seen in mammals (Matsuoka et al., 1995; Galli et al., 2002; Youdim et al., 2002; Wang et al., 2006). *Lymnaea* is an ideal model with which to test the mechanism by which flavonoids alter cognition. The neuronal circuit that drives aerial respiratory behaviour consists of only three interneurons (Syed et al., 1992), of which RPeD1 (the neuron initiating aerial respiratory behaviour) is absolutely necessary for LTM formation, reconsolidation and extinction (Spencer et al., 1999; Scheibenstock et al., 2002; Spencer et al., 2002; Sangha et al., 2003a; Sangha et al., 2003c). RPeD1 excitability also predicts strain-specific variations in the ability to form LTM (Braun et al., 2012). 'Smarter' *Lymnaea* strains in their naive state exhibit decreased RPeD1 excitability, i.e. they appear to be in a 'primed' state to form LTM. Our prediction, to be tested in the future, is that exposure to epi results in this 'primed' RPeD1 state, such that snails have an enhanced ability to form LTM. Whether other behaviours in *Lymnaea* that undergo associative learning and LTM formation (e.g. appetitive conditioning of feeding) will be similarly affected by epi also remains to be determined.

Our findings presented here provide the groundwork for future molecular analysis of how epi acts at the neuronal level and the mechanisms involved in the alterations to memory formation, storage and extinction.

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