© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 816-823 doi:10.1242/jeb.130765

RESEARCH ARTICLE

A flavanoid component of chocolate quickly reverses an imposed memory deficit

Bogdan Knezevic, Yoshimasa Komatsuzaki, Emily de Freitas and Ken Lukowiak*

ABSTRACT

The ability to remember is influenced by environmental and lifestyle factors, such as stress and diet. A flavanol contained in chocolate, epicatechin (Epi), has been shown to enhance long-term memory (LTM) formation in *Lymnaea.* Combining two stressors (low-calcium pond water and crowding) blocks learning and all forms of memory; that is, this combination of environmentally relevant stressors creates a memory-unfriendly state. We tested the hypothesis that Epi will immediately reverse the memory-unfriendly state, i.e. that snails in the memory-deficit state when trained in Epi will immediately become competent to learn and form memory. We found that Epi not only reverses the memory-deficit state but also further enhances LTM formation. Thus, a naturally occurring bioactive plant compound can overcome a memory-unfriendly state. This supports the idea that bioactive substances may mitigate memory-making deficits that, for example, occur with ageing.

KEY WORDS: Long-term memory, Epicatechin, Stress, *Lymnaea*

INTRODUCTION

Memory is what makes you you, and me me (Milner et al., 1998). How we learn and form memory is thus extremely important for us to understand. We know that lifestyle choices (e.g. diet) and our immediate environment (e.g. various stressors) play important roles in enhancing or blocking memory formation and its recall. Importantly, we are all also aware of the devastating effects that individuals and their families endure when a person no longer has the ability to learn and remember. The search for remedies to prevent or to mitigate the devastating effects of memory forming loss is a priority of biomedical research. Previously, we have shown that it is possible to block learning and all forms of memory in our *Lymnaea* model system, by subjecting the animals to a combination of stressors (crowding and a low-calcium environment) before operant conditioning (Dalesman et al., 2013). Here, we tested the hypothesis that a naturally occurring substance, the flavanol (−) epicatechin (Epi), will overcome the aforementioned memory blockade. There are a number of epidemiological studies suggesting that the intake of cocoa flavanols (CFs) such as Epi are correlated with a lower incidence of cognitive impairment (Kuriyama et al., 2006) and significantly better cognitive performance (Letenneur et al., 2007; Nurk et al., 2009). However, the mechanism(s) by which the CFs bring about their effects on memory are not clear. As cocoa is a good source of Epi, it may be of interest (perhaps only to us) that Linnaeus named both the snail we use (*Lymnaea stagnalis*) and the cocoa plant (*Theobroma cacoa*). Perhaps Linnaeus anticipated findings some 260 years after assigning the scientific name to cocoa, *Theobroma*, which roughly translates to ‘food of the gods’.

We tested our hypothesis in our simple model system, aerial respiratory behaviour in the pond snail *Lymnaea* (Lukowiak et al., 1996, 1998, 2000). Our preparation has a number of attractive attributes including that it can be operantly conditioned, a form of associative learning; following conditioning, short (STM), intermediate (ITM) and long-term memory (LTM) form (Lukowiak et al., 1996, 2000; Dalesman and Lukowiak, 2012). In addition, we have demonstrated that: (1) memory recall is context specific (Haney and Lukowiak, 2001); (2) one-trial learning occurs (Martens et al., 2007); (3) reconsolidation and extinction occur (Sangha et al., 2003a,b); (4) forgetting is an active process (Sangha et al., 2005); and (5) a false memory can be implanted into the snail following activation of the memory (Lukowiak et al., 2007). We have also shown that: (1) a single neuron, RPeD1 is a necessary site for LTM formation (Scheibenstock et al., 2002) and significant changes in synaptic input as well as changes in neuronal excitability of this neuron have been correlated with LTM (Spencer et al., 1999, 2002; Braun and Lukowiak, 2011); (2) learning and memory formation can be demonstrated in an *in vitro* semi-intact preparation (McComb et al., 2005); (3) differences in cognitive ability occur at both the behavioural (Orr et al., 2009; Dalesman et al., 2011a,b,c) and neuronal levels (Braun and Lukowiak, 2011; Braun et al., 2012); and (4) a partial proteomics profile exists for changes underlying LTM formation (Rosenegger et al., 2010). Finally, a number of environmentally relevant stressors either enhance or block memory formation (Lukowiak et al., 2008, 2010, 2014).

Lymnaea satisfies its respiratory requirements bi-modally. That is, in eumoxic conditions it mainly relies on cutaneous respiration, while in hypoxic conditions it utilizes aerial respiration. Thus, it is possible to train snails not to perform aerial respiration without negatively impacting their health. Aerial respiratory behaviour is driven by a 3-neuron central pattern generator (CPG) whose necessity and sufficiency has been experimentally verified (Syed et al., 1990, 1992). In our operant conditioning procedure, we train snails not to perform aerial respiration in a situation where this behaviour should predominate. If we combine two stressors (crowding and low environmental Ca^{2+}) that independently block only LTM formation, leaving intact STM and ITM (De Caigny and Lukowiak, 2008; Knezevic et al., 2011; Dalesman and Lukowiak, 2010; Dalesman et al., 2011a,b,c), we are able to completely block all forms of memory (i.e. STM, ITM and LTM; Dalesman et al., 2013).

We have also shown that training snails in Epi-supplemented pond water enhances LTM formation (Fruson et al., 2012). The Epi-enhanced LTM formed faster, persisted longer and was more resistant to extinction. Moreover, Epi did not alter other behavioural tests (locomotion, baseline breathing rates, etc.), thus exemplifying

Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1.

*Author for correspondence (lukowiak@ucalgary.ca)

Received 20 August 2015; Accepted 30 December 2015

Journal of Experimental Biology (2016) 219, 816-823 doi:10.1242/jeb.130765

specificity for the drug to interact with the memory-forming neuronal pathways essential for LTM formation (Fruson et al., 2012). More interesting in regards to stress exposure of *Lymnaea*, Epi was able to overcome the suppressive effects of low environmental Ca^{2+} on LTM formation (Knezevic and Lukowiak, 2014). That is, while even a 1 h exposure to low-calcium pond water before the initiation of training is sufficient to block LTM but not ITM, training snails in Epi and the low-calcium pond water results in LTM formation.

Here, we tested the hypothesis that Epi can overcome the effects that crowding together with low calcium has on all forms of memory formation in *Lymnaea*.

MATERIALS AND METHODS

Animals

Adult pond snails, *Lymnaea stagnalis*, 25±1 mm spire height, were obtained from a stock derived from original stocks at the Vrije Universiteit, Amsterdam. We refer to these as *W-strain* snails. Adult snails were raised in a tank containing artificial pond water (containing $0.26\ g\ l^{-1}$ Instant Ocean, Spectrum Brands Inc., Madison, WI, USA, and calcium sulphate dihydrate added so that the Ca^{2+} concentration was $80\ mg\ l^{-1}$), at the University of Calgary. For the low-calcium experiments, 1 week prior to operant conditioning, snails were transferred to oxygenated artificial pond water with a Ca^{2+} concentration of $20\ mg\ l^{-1}$. This is referred to as the low-calcium environment. Tanks were maintained at a temperature of 20±1°C, and snails were normally housed at density of 1 snail per litre. Romaine lettuce was provided *ad libitum*. Snails were then transferred from these conditions into smaller containers, where calcium levels and/or drug exposure was altered, as discussed below. All other conditions (temperature, density, food) remained unchanged.

Training procedure

Operant conditioning training sessions (TSs) were 0.5 h in duration as were the memory test (MT) sessions and were conducted in hypoxic pond water. Thus, when we measured whether memory occurred (see below), we in reality used a ‘savings’ test; and the MT session served as another training session (see below). Pond water was made hypoxic by bubbling with nitrogen for 20 min; snails were transferred into a 1 litre beaker containing 500 ml of this hypoxic water. The periods of rest between training sessions (1 h) as well as leading up to the MT sessions were in eumoxic pond water at the specified calcium concentration (standard and low calcium). Training consisted of applying a tactile stimulus to the pneumostome (the snails’ respiratory opening) as the snail began to open it. The tactile stimulus (i.e. poke) caused the pneumostome to close. We thus recorded the total number of pokes (representing attempted pneumostome openings) for each snail during the training and memory testing sessions. Typically, 10 snails, each individually labelled, were trained in a beaker at the same time (Lukowiak et al., 1996).

Memory (i.e. STM, ITM and LTM) in our model system used here has been operantly defined (Lukowiak et al., 2000). We defined memory as having been formed in snails trained with the two 0.5 h training session procedure (with a 1 h interval between sessions) if the number of attempted openings in MT (either the 3 h or 24 h test) is significantly fewer than in TS1 and not significantly greater than in TS2 (Lukowiak et al., 1998). When the single 0.5 h training session procedure is used, LTM is defined as the number of attempted openings in MT being significantly lower than in TS1 (Martens et al., 2007).

Snail maintenance procedures used in the various training groups

A group of naive snails (*N*=18; Fig. 1A) was trained (i.e. operantly conditioned) in $15\ mg\ l^{-1}$ Epi (Sigma-Aldrich, St Louis, MO, USA) added to standard pond water ($80\ mg\ l^{-1}\ Ca^{2+}$) and then tested for LTM in standard pond water. They received a single 0.5 h training session (TS1) and then were tested for LTM 24 h later (MT) in standard pond water. They were maintained in standard pond water during the 24 h interval between training and memory testing. A second naive group of snails (Fig. 1B; *N*=13) received a single 0.5 h TS in standard pond water (i.e. no Epi) and memory was tested 24 h later.

A third group of naive snails (Fig. 1C; *N*=18) was kept in low-calcium ($20\ mg\ l^{-1}$) conditions for 1 week. Snails were then crowded for 1 h (20 snails/100 ml), before being transferred to a testing beaker, where they received a 0.5 h training session (TS1). Following TS1, they were returned to their home aquaria (still low calcium), before receiving a second 0.5 h training session (TS2). The snails were then transferred to a eumoxic aquarium for 3 h, where they were tested (3 h MT) for ITM. Following the 3 h memory test session, the snails were once again transferred to their home aquaria for 24 h. These snails were then tested for LTM (24 h MT).

Low-Ca^{2+}, crowded and Epi ITM experimental group

A different group of naive snails (Fig. 2; *N*=35) was kept in low-calcium ($20\ mg\ l^{-1}$) conditions for 1 week. Snails were then crowded for 1 h (20 snails/100 ml), before being transferred to a testing beaker containing Epi ($15\ mg\ l^{-1}$ in low-calcium pond water), where they received two 0.5 h training sessions in the low-calcium plus Epi pond water separated by a 1 h interval. During the 1 h intersession interval, they were in the low Ca^{2+} environment without Epi. We then randomly picked 17 snails and tested them for memory 3 h after TS2. This is a test for ITM and occurred in the low-calcium environment (i.e. no Epi was present). The remaining 18 snails were tested for memory 24 h after TS2. Again Epi was not present in the low-calcium environment for this memory test. Thus, each snail was only tested for memory once.

Low-Ca^{2+}, crowded and Epi LTM experimental group

Another group of naive snails (Fig. 3; *N*=16) was kept in low-calcium conditions for 1 week. Snails were then crowded for 1 h, before being transferred to a testing beaker, where they received two 0.5 h training sessions separated by a 1 h interval. The training sessions were performed in the low-calcium and Epi environment ($15\ mg\ l^{-1}$ in low-calcium pond water). We tested these snails for LTM 96 h later in the low-calcium environment but without added Epi (MT1). Following this session, snails were returned to a normal calcium environment. They were then assessed for memory in standard pond water 72 h later (MT2). Finally, they were again returned to their home aquaria (normal calcium environment) for 1 week (168 h) before being tested for memory again (MT3).

Standard Ca^{2+} and Epi control LTM experimental group

The penultimate group of naive snails (Fig. 4A; *N*=11) maintained in standard pond water received two 0.5 h training sessions separated by a 1 h interval. They were then tested for LTM 96 h later (MT1). Following this memory test, snails were again tested for memory (MT2) 72 h later and then a final memory test (MT3) was performed 1 week (186 h) later.

The final cohort of naive snails (Fig. 4B; *N*=18) was treated exactly like the penultimate cohort, except that the first two 0.5 h training sessions were performed in Epi. All other memory testing sessions were in pond water without Epi.

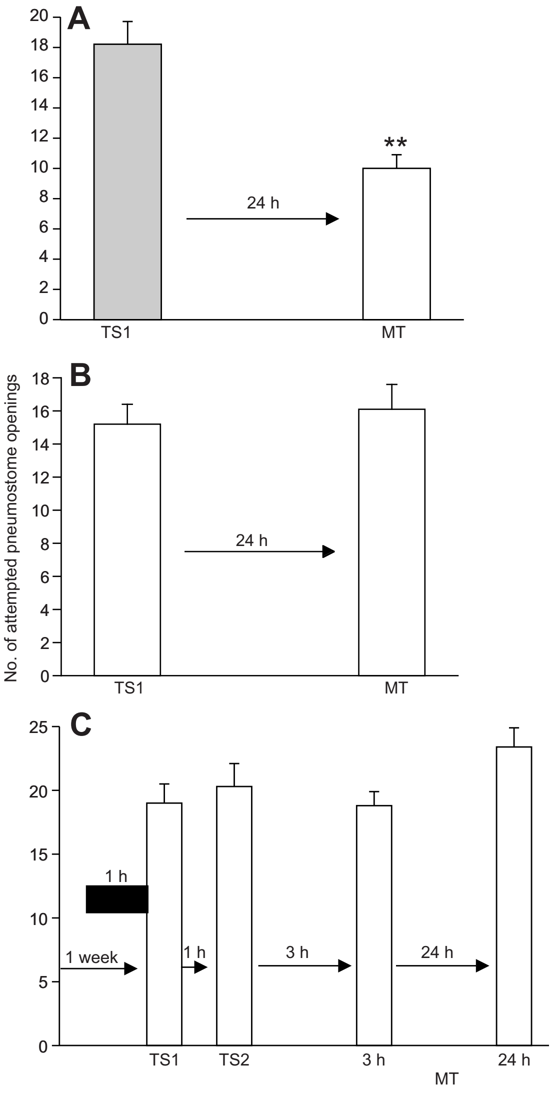

Fig. 1. The effects of epicatechin-supplemented standard pond water on memory enhancement and the blocking of learning and memory by the combination of crowding and low-calcium pond water. (A,B) Mean (+s.e.m.) number of attempted pneumostome openings during a single 0.5 h training session (TS1) and a memory test (MT) 24 h after TS1. (A) Training snails (N=18) in epicatechin (Epi)-supplemented pond water (grey bar) caused enhanced long-term memory (LTM) formation. The number of attempted pneumostome openings in MT was significantly lower than in TS1 (paired t-test, t=4.086, $**P<0.01$). (B) Training snails (N=13) in standard pond water without Epi did not result in LTM formation. The number of attempted openings in MT was not significantly lower than in TS1 (paired t-test, t=0.856, P>0.05). (C) Mean (+s.e.m.) number of attempted pneumostome openings in snails (N=18) maintained in low-calcium (20 mg l^{-1}) pond water 1 week prior to training and during the remainder of the experiment. Snails were also crowded for 1 h before TS1 (black bar). Learning, ITM and LTM were not observed. That is, the number of attempted openings in TS2, and in MTs 3 h (i.e. ITM) and 24 h (i.e. LTM) later was not significantly lower than that in TS1. Thus, the criteria for memory were not met. Notice also that even though these snails received an additional training session 3 h after TS2 (i.e. the 3 h MT), LTM was not observed. [ANOVA, $F_{3,51}$=1.534, mean squared error (MSE)=37.952, P=0.217.]

Statistical analyses

Data were analysed separately in SPSS Statistics v20 (SPSS Inc., Chicago, IL, USA). A repeated-measures ANOVA was used to compare the mean number of attempted pneumostome openings across training and memory test sessions. Homogeneity of variance was confirmed using Mauchly's test for sphericity before analysis. When tests yielded an overall significance, *post hoc* paired t-tests were used to determine between which trials (TS1, TS2 or MT) the significant difference occurred. In the experiments with and without Epi added to the pond water, involving only a single 0.5 h training session and a single memory test 24 h later, a paired t-test was used to determine whether memory formed.

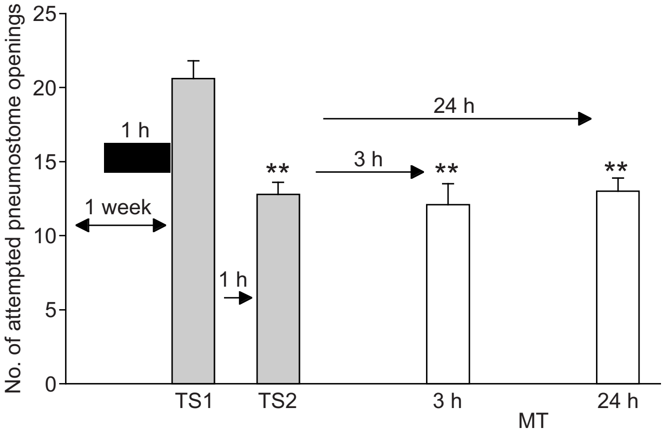

Fig. 2. Training in Epi-supplemented pond water reverses stress-imposed learning and memory deficits. The stressors were low calcium for 1 week prior to training and crowding for 1 h before TS1 (black bar). Plotted are the mean (+s.e.m.) number of attempted pneumostome openings of snails (N=35) during two 0.5 h training sessions in epi-supplemented low-calcium pond water (TS1 and TS2, grey bars); snails received a MT either 3 h (N=17) or 24 h (N=18) later. Both the 3 h and the 24 h MT were performed in the absence of Epi in the low-calcium pond water. As can be seen, learning, ITM and LTM occurred (i.e. the number of attempted pneumostome openings in TS2 was significantly lower that that in TS1; and the number of attempted openings in both the 3 h and the 24 h MT was significantly lower than that in TS1, but not significantly greater than that in TS2) (ANOVA, $F_{2,32}$=11.809, MSE=42.373, $**P=0.01$).

RESULTS

We first wished to replicate previous experiments to show that: (1) Epi enhances LTM formation; and (2) the combination of a low-calcium pond water environment and 1 h of crowding immediately prior to operant conditioning training blocks all forms of memory in *Lymnaea*. We used three different cohorts of naive snails to replicate these previous findings.

As can be seen in Fig. 1A, snails (N=18) that received a single 0.5 h training session in standard Epi-supplemented pond water (i.e. 15 mg l^{-1} Epi in 80 mg l^{-1} Ca^{2+}) exhibited LTM when tested 24 h later. That is, the number of attempted pneumostome openings in MT was significantly lower (P<0.001) than the number of attempted openings in TS1. In a second cohort (Fig. 1B; N=13), snails receiving a single 0.5 h training session in standard pond water (i.e. without Epi) did not form LTM. Finally, we used a third cohort of naive snails (Fig. 1C; N=18) that were maintained for 1 week in the low-calcium pond water (20 mg l^{-1} Ca^{2+}) and then crowded (20 snails/100 ml) for 1 h just prior to operant conditioning training. These snails received two 0.5 h training sessions, separated by a 1 h interval during which they continued to be maintained in the low-calcium pond water but were not crowded. In this cohort of snails, we found the following: (1) there was no evidence of learning (i.e. the number of attempted pneumostome openings in TS2 was not significantly lower than in TS1); (2) ITM was not present (i.e. when we tested whether snails had memory 3 h after TS2 we found that

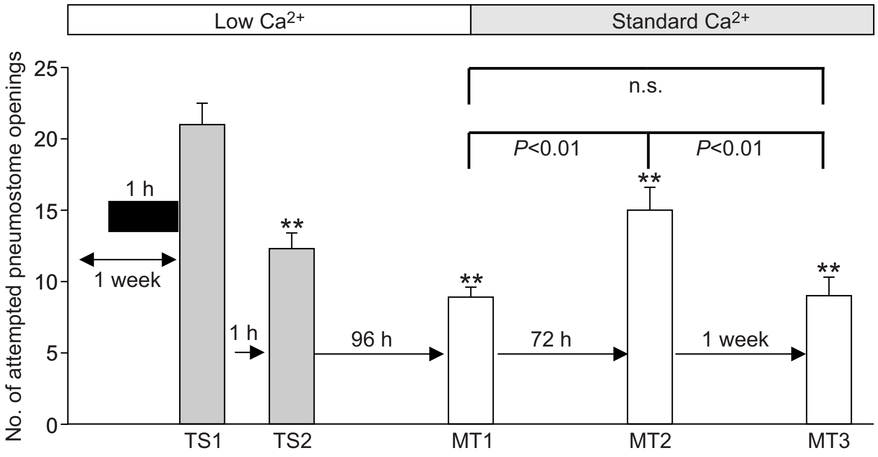

Fig. 3. Training in Epi-supplemented pond water results in long-lasting LTM even in snails exposed to the memory-unfriendly combined stressors. Naive snails (N=16) were subjected to the same low-calcium and crowding procedures as above. Plotted are the mean (+s.e.m.) number of attempted pneumostome openings for two 0.5 h training sessions (TS1 and TS2) in Epi-supplemented pond water (grey bars). Memory tests were performed in the low-calcium pond water (i.e. no Epi) 96 h following TS2 (MT1). Snails were then transferred to standard calcium (i.e. 80 mg l^{-1}) pond water, and memory tests were carried out 72 h after MT1 (MT2) and 1 week after MT2 (MT3). Both learning and memory occurred (ANOVA, $F_{4,60}$=21.673, MSE=18.900, **P=0.01 compared with TS1). n.s., not significant.

there was no significant difference between the 3 h MT and TS1 or, for that matter, TS2; (3) when tested 24 h later (i.e. 24 h MT), there was no evidence of LTM in that the number of attempted openings in the 24 h MT session was not significantly lower than in TS1. Importantly, LTM was not observed in the 24 h MT even though the 3 h MT test is actually another training session (i.e. no LTM after three training sessions), showing that this cohort of snails experiencing both the low-calcium and crowded stressors has an inability to learn and form ITM and LTM.

Having shown that maintaining snails in low-calcium pond water and then crowding them for 1 h just prior to subjecting them to the operant conditioning procedure blocked memory formation (Fig. 1C), we asked whether training in Epi-supplemented low-calcium pond water would mitigate this memory-forming deficit. We therefore used a new cohort of naive snails (N=35; Fig. 2), exposed them to the two stressors and then trained them in Epi-supplemented low-calcium pond water. As can be seen, the results for TS2 were significantly different from those for TS1, indicating that learning had occurred. We then tested 17 of these snails 3 h later for ITM (3 h MT) and the remaining 18 snails 24 h later for LTM (24 h MT). Snails trained with similar exposure to the stressors but without Epi did not exhibit learning (Fig. 1). When we examined whether ITM was present 3 h after TS2, we found that memory had formed. In a similar manner, when we examined whether LTM was present in the snails 24 h after they experienced TS2, we found that LTM was present. That is, the number of attempted openings in the 24 h MT session was significantly lower than in TS1 and not significantly greater than in TS2. Thus, the criteria for both ITM and LTM formation were met. It is important to note that Epi-supplemented pond water was not used in either the 3 h or the 24 h memory test.

Having shown that Epi enabled snails to make both ITM and LTM in an environment that prevents these types of memory formation, we wanted to know whether the LTM formed as a result

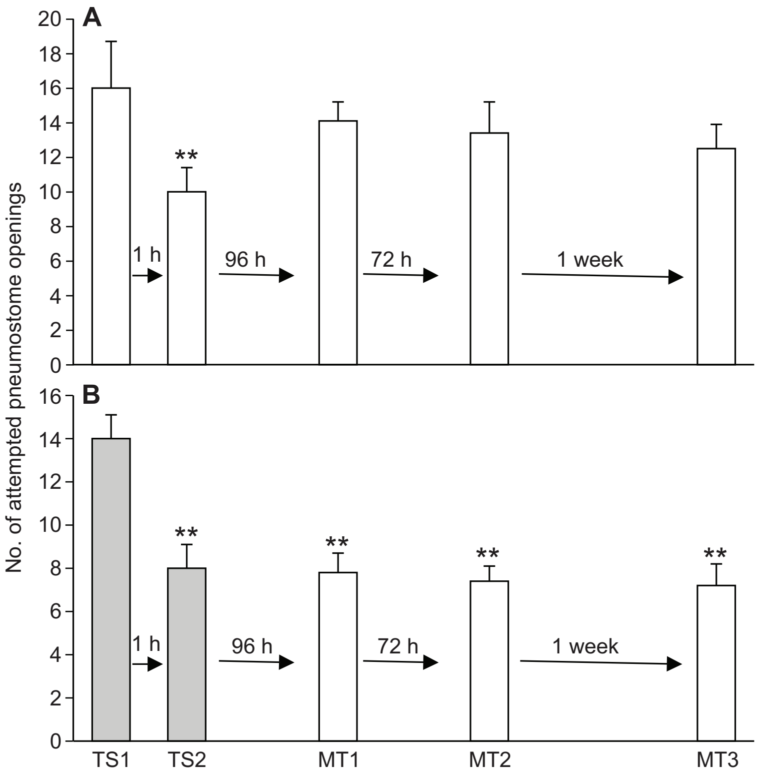

Fig. 4. Training in Epi-supplemented standard pond water causes long-lasting enhancement of LTM. (A) Snails (N=11) trained in standard pond water exhibited learning (the number of attempted openings in TS2 was significantly lower than that in TS1; **P=0.01) but memory was not exhibited in any of the MTs (i.e. the number of attempted openings in MT1, MT2 and MT3 was not significantly different from that in TS1). (B) As in A, except that snails (N=16) received the two 0.5 h training sessions (TS1 and TS2) in Epi-supplemented standard pond water (grey bars). Both learning and memory were formed (ANOVA, $F_{4,60}$=6.957, MSE=21.11, **P<0.01). That is, in all MT comparisons, the number of attempted openings was significantly lower than in TS1 (P<0.01) and not significantly greater than in TS2. The Epi-enhanced LTM persisted for at least 96 h following TS2. Subsequent testing showed that memory duration could be extended, even in standard pond water, where forgetting can occur.

of training in Epi persists for longer than 24 h. Thus, another cohort of naive snails (*N*=16; Fig. 3) was subjected to the same unfriendly memory-forming environment but trained (i.e. TS1 and TS2) in Epi-supplemented pond water. Again, we saw an immediate significant decrease in the number of attempted openings in TS2 compared with TS1. Following TS2, snails were returned to the low-calcium environment aquaria for 96 h (i.e. no Epi). We then tested for the presence of memory and found that the number of attempted openings in MT1 (i.e. the 96 h memory test, Epi not present) was significantly lower than in TS1 and not significantly different from that in TS2. Thus, training in Epi even in an unfriendly memory-formation environment is sufficient to result in a 96 h LTM. Following the 96 h MT session, we transferred the snails to the standard calcium (80 mg l^{-1}) environment and 72 h later tested snails for memory (i.e. MT2). We performed this experiment to see whether the memory would deteriorate (i.e. forgetting) as it does in standard pond water (Knezevic et al., 2011). As can be seen in Fig. 3, the number of attempted openings in MT2 was significantly greater than that in MT1. While the number of attempted openings in MT2 was significantly lower than the number in TS1, the criteria for LTM were not met because the number of attempted openings in MT2 was significantly greater than in MT1. That is, the operational definition of LTM (Lukowiak et al., 1996) was not met. Following MT2, we waited an additional week and performed another memory test (MT3). The number of attempted pneumostome openings in MT3 was significantly lower than in MT2 and TS1 and not significantly greater than the number in MT1. Thus, LTM was present. That is, it appears that while LTM was not present according to the operational definition of LTM, there was a residual memory trace present (Parvez et al., 2005, 2006) such that the MT2 session (it is also operationally a training session) built upon this trace to cause LTM to form in MT3.

Because the data obtained in the experiment described in Fig. 3 are complicated and possibly difficult to interpret, we performed two additional experiments. These experiments are shown in Fig. 4. In Fig. 4A, a naive cohort of snails (*N*=11) was maintained and trained in standard pond water (i.e. 80 mg l^{-1} Ca^{2+}). There was a significant decrease in the number of attempted pneumostome openings in TS2 compared with TS1, indicating that ITM had formed. However, LTM was not present 96 h later. That is, the number of attempted openings in MT1 was not significantly lower than in TS1. This experiment confirms previous data that the memory formed using this procedure in this strain of snails does not persist for 96 h. The snails were then returned to their home aquaria and tested for memory 72 h later (i.e. MT2). Again, memory was not observed. Finally, after a further 1 week interval, memory was again tested (MT3) and was found not to be present. Thus, in standard pond water this training procedure did not result in a long-lasting (i.e. >24 h) LTM. There also did not appear to be any residual memory trace for the subsequent memory test session (i.e. MT2) to build upon to cause the formation of a 1 week LTM.

In Fig. 4B, a similar training procedure was used on snails maintained in standard pond water. However, this naive cohort of snails (*N*=18) received the two training sessions (TS1 and TS2) in Epi-supplemented pond water. Again, in TS2 there were significantly fewer attempted pneumostome openings than in TS1, indicating that ITM formed. Following TS2, snails were returned to their home aquaria for 96 h before being tested for memory (i.e. MT1). As can be seen in Fig. 4B, LTM was present. That is, the number of attempted pneumostome openings in MT1 was significantly lower than in TS1 and not significantly greater than in TS2. The snails were again returned to their home aquaria and tested for LTM 72 h later (MT2). As before, LTM was present. We then returned these snails to their home aquarium and tested for memory 1 week later; memory was present in MT3. Thus, training snails for two sessions in Epi resulted in long-lasting memory.

DISCUSSION

A clear advantage of our *Lymnaea* model system is that we are able to completely block the ability to learn and form memory by using a specific combination of environmentally relevant stressors before operant conditioning training in snails that are typically competent to learn and form memory. This allows us to easily put an animal in a ‘memory-unfriendly’ state and then determine procedures or bioactive compounds that mitigate the negative cognitive effects inflicted on the snail by the stressors. Here, we demonstrate that training in Epi, a naturally occurring food substance, quickly reverses the total inability to learn and form memory. That is, when trained in the presence of Epi, the animals immediately regain the ability to associatively learn and form ITM and LTM. This finding gives hope that it is possible to devise strategies (see below) making use of natural products to mitigate learning and memory deficits.

We first showed that Epi has the ability to enhance LTM formation in non-stressed animals (Fig. 1). That is, whereas in our *W-strain* snails it takes at least two 0.5 h operant conditioning training sessions separated by a 1 h interval to result in LTM, a single 0.5 h training session in Epi-supplemented pond water is sufficient to cause LTM formation. These data are in accord with the original finding of Fruson et al. (2012).

We then demonstrated that a combination of low calcium and crowding, which both energetically and emotionally tax the snail, result in a complete blockage of learning and memory processes (STM, ITM and LTM), confirming our earlier published work (Dalesman et al., 2013). Memory formation is an energetically demanding process, requiring both new protein synthesis and altered gene activity (Sangha et al., 2003c; Parvez et al., 2005). As both calcium deprivation and crowding are stressors that force *Lymnaea* to use resources significantly more sparingly (Dalesman and Lukowiak, 2010), it follows that a necessary husbanding of resources takes precedence over higher order and energetically expensive functions like new memory formation. This indicates that there is a ‘cost’ to memory formation. It may also be that these two potent stressors together create an emotional state that is incompatible with memory formation. We posit that it is possible to establish such an emotional state in *Lymnaea*. Damasio (2010) has written: ‘in simple organisms capable of behavior but without a mind process, emotions can be alive and well…’. That is, changes in the ability to form memory occur as a result of subjecting an animal to certain stressors or combinations of them, resulting in the creation of an emotional state, which significantly alters the ability to form or recall memory.

To put it in another context, the combination of stressors used here pushes the organism to the far right on the so-called Yerkes–Dodson (Y–D) curve. This ‘Y–D law’ can be used to describe the effect of stress on learning and memory, stating that at different stress levels the ability to form memory changes. In textbooks this ‘law’ is shown as an inverted-U function. It should be noted that the Yerkes and Dodson (1908) paper did not present such an inverted-U curve. The inverted-U function is actually a figure adapted from Donald Hebb’s 1955 presidential address to the American Psychological Association (Hebb, 1955; see also Diamond et al., 2007; Ito et al., 2015a; Kojima et al., 2015). It appears that Hebb was unaware of the earlier Yerkes and Dodson paper (Diamond et al., 2007). Hebb hypothesized that with too little or too much stress, learning and memory formation are not optimal, hence the inverted-U curve. Thus, the stressors we used here ultimately result in changes in the neuronal circuit meditating memory formation that are incompatible for memory formation. What these changes are remain to be determined.

While we had previously shown Epi to enhance LTM formation (Fruson et al., 2012), our present findings go well beyond this as we now show that Epi quickly and effectively reverses a behavioural state where neither learning nor memory occurs. This is a significant finding. Thus, with only two 0.5 h training sessions in Epi-supplemented low-calcium pond water, both ITM and LTM are formed (Fig. 2) in the situation where all forms of memory are blocked in the absence of Epi. Moreover, in the learning and memory deficit state caused by the two stressors, the two 0.5 h training sessions in Epi resulted in a LTM that persisted for at least 96 h (Fig. 3). In a typical experiment in standard pond water in the *W-strain* snails, two 0.5 h training sessions separated by a 1 h interval resulted in a 24 h but not a 48 h LTM (Sangha et al., 2003c). Thus, not only did Epi-supplemented pond water cause the animals to revert to a state where memory could be formed but also an enhanced memory-forming state was achieved. These findings are consistent with the hypothesis that training in Epi-supplemented pond water results in a 'super' memory state. In such a state, forgetting is delayed and the extinction process is impeded (Fruson et al., 2012). Of further importance is the fact that Epi does not have to be present in the memory test session for memory to be recalled. That is, while LTM in *Lymnaea* is context specific (Haney and Lukowiak, 2001) the boost in LTM formation caused by training in Epi results in memory that can be recalled without the presence of Epi. How this memory boost is reflected at the neuronal level in the circuit that drives aerial respiratory behaviour is now being investigated.

In Fig. 3, after showing that Epi reversed the inability to form both ITM and LTM in the memory-unfriendly environment (i.e. crowding and low calcium), we showed that the resulting LTM persisted for at least 96 h after training. We then asked what would happen to this memory when snails were returned to standard pond water (i.e. 80 mg l^{-1} Ca^{2+}). When we tested for LTM after 72 h in the standard conditions, we found that LTM was not present as the number of attempted pneumostome openings in the MT2 session was significantly greater than the number of attempts in MT1 ($P<0.01$). Based on our operational definition of LTM (Lukowiak et al., 1996), LTM was not present even though the number of attempted openings in MT2 was significantly lower than in TS1. These data show that returning snails to standard conditions allows forgetting to occur. Forgetting is blocked in the low-calcium environment (Knezevic et al., 2011; Karnik et al., 2011), as is LTM formation. Both this LTM formation and forgetting require altered gene activity and new protein synthesis (Sangha et al., 2005). Notice, however, that when we then tested snails 1 week later, memory was present. We posit that the memory exhibited in MT3 was the result of the training that occurred during the MT2 session acting on a 'residual' memory trace arising from training in Epi that was present in the MT2 session leading to a very long-lasting LTM (Parvez et al., 2005, 2006).

To better support the hypothesis that the memory exhibited in MT3 shown in Fig. 3 was the result of an enhanced memory formation process arising from training in Epi-supplemented pond water, we performed two additional experiments (Fig. 4). These data show that in *W-strain* snails trained in two 0.5 h training sessions in standard pond water, LTM does not persist for 96 h; nor do these data show that there is a residual memory trace present 72 h after MT1 which allows LTM to be present 1 week after MT2. That is, LTM is not present in MT3. However, when *W-strain* naive snails were trained with two sessions in Epi-supplemented normal pond water, we found LTM not only 96 h later but also 72 h after MT1 and 1 week after MT2. These data are all consistent with the hypothesis that training snails in Epi-supplemented pond water results in a very long-lasting memory that is resistant to forgetting and that is not dependent on Epi being present in the memory recall sessions.

The finding that Epi quickly reverses the 'non-memory' conducive state that was brought about by the two stressors gives hope that naturally occurring substances can be used to reverse states that are not favourable for memory formation. However, the literature is filled with claims of remarkable findings concerning natural products and memory enhancement drugs that do not seem to work out in the real world. Here, we are not suggesting that Epi is a 'wonder-drug', only that in our model system it has the ability to quickly change the state of the system from one not conducive to memory formation to one extremely conducive to memory formation. It may well be that this compound only 'works' in the manner we described on certain stress-related states that block memory formation. Our data show that Epi not only enhances learning and memory formation in snails capable of learning and forming memory but also can overcome a state of memory formation impediment. With the establishment of behavioural effects like those shown here, we can move to examining the mechanisms at a cellular and molecular level to elucidate the causal neuronal changes underlying these specific behavioural states. It may then become possible to begin to translate such knowledge from our animal model to the clinic, for potential use to mitigate states that are not conducive to memory formation.

While our data in *Lymnaea* suggesting that a flavanoid such as Epi can enhance memory formation, even under situations where the formation of memory is severely limited, is of interest to molluscan neurobiologists, it will only be of interest to the wider neurobiology community if these naturally occurring substances play similar roles in humans. It appears that there are similarities to our work in the literature dealing with mood and cognition in humans. There is a positive correlation based on a number of epidemiological studies between the intake of flavanoids such as Epi (contained in dark chocolate) and a lower incidence of cognitive impairment (Kuriyama et al., 2006) and significantly better cognitive performance in subjects without dementia (Letenneur et al., 2007; Nurk et al., 2009). More recently, Mastroiacovo et al. (2015) provided further evidence that daily consumption of CFs improved cognitive function in healthy, non-demented elderly individuals. Subjects (~100 individuals) were randomly assigned into one of three groups that received a daily drink containing low, intermediate or high amounts of CFs. Their results showed that in elderly people not suffering from cognitive dysfunction, regular intake of CFs improved measures of cognitive performance and that the effects on cognition appeared to be dependent on the amount of CF. Interestingly, the authors suggested that the largest contribution to cognitive improvements came from changes brought about by the CFs action to improve insulin sensitivity. Previously, Grassi et al. (2008) showed that the ingestion of dark chocolate, which contains CFs, reduced blood pressure and increased insulin sensitivity in glucose-intolerant, hypertensive subjects. In addition, it was also shown that CFs in cocoa induce vasodilation of the peripheral and cerebral vascular system (Sorond et al., 2008; Francis et al., 2006), increasing brain blood flow and perfusion mainly through an improvement in nitric oxide bioavailability in endothelial cells

(Heiss et al., 2010). Thus, in humans, CF-containing foods can be effective in protection against the development of age-related cognitive dysfunction, possibly reversing or with a restorative effect on certain aspects of age-related cognitive decline (Field et al., 2011; Morris, 2012). It is also worth noting here that insulin has positive effects on LTM formation in *Lymnaea* (Murakami et al., 2013a,b; Hatakeyama et al., 2014; Ito et al., 2015b). Whether Epi has an effect on insulin-like molecules in *Lymnaea* remains to be determined.

While we have not yet elucidated the mechanism by which Epi enhances LTM formation in our model system, we know that this water-soluble substance quickly (within 30 min) crosses the skin membrane of the snail, and as the snails possess an open circulatory system, Epi can directly contact CNS neurons. While it has been suggested that Epi may enhance cognitive function in humans by increasing cerebral blood flow (van Praag et al., 2007; Brickman et al., 2014), this is unlikely to account for improved memory in *Lymnaea* as it possesses an open circulatory system. Epi does not remove the stressors; rather, it overcomes the negative effects of the stressors on memory formation. This may be considered analogous to the anxiolytic effect that dark chocolate has according to some reports in humans (Nehlig, 2012). Our findings suggest that through ingesting foods rich in CFs, we may be able to overcome states such as ageing where memory formation and its retention are sometimes compromised. These results provide the basis of future studies in *Lymnaea* in order to elucidate how dietary substances such as CFs cause changes in neurons, which play a necessary role in memory formation, in an induced state where learning and memory do not occur.

Competing interests

The authors declare no competing or financial interests.

Author contributions

B.K. performed the majority of the experiments and contributed to the writing of the paper; Y.K. performed a number of the experiments; E. de F. performed a number of the control experiments; K.L. designed the experiments and wrote and edited the paper.

Funding

This research was funded by the Natural Sciences and Engineering Research Council of Canada.

References

Braun, M. H. and Lukowiak, K. (2011). Intermediate and long-term memory are different at the neuronal level in *Lymnaea stagnalis* (L.). *Neurobiol. Learn. Mem.* **96**, 403-416.

Braun, M. H., Lukowiak, K., Karnik, V. and Lukowiak, K. (2012). Differences in neuronal activity explain differences in memory forming abilities of different populations of *Lymnaea stagnalis. Neurobiol. Learn. Mem.* **97**, 173-182.

Brickman, A. M., Khan, U. A., Provenzano, F. A., Yeung, L.-K., Suzuki, W., Schroeter, H., Wall, M., Sloan, R. and Small, S. (2014). Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. *Nat. Neurosci.* **17**, 1798-1803.

Dalesman, S. and Lukowiak, K. (2010). Effect of acute exposure to low environmental calcium on respiration and locomotion in *Lymnaea stagnalis* (L.). *J. Exp. Biol.* **213**, 1471-1476.

Dalesman, S. and Lukowiak, K. (2012). How stress alters memory in 'Smart' snails. *PLoS ONE* **7**, e32334.

Dalesman, S., Rundle, S. D. Lukowiak, K. (2011a). Microgeographical variability in long-term memory formation in the pond snail, *Lymnaea stagnalis*. *Anim. Behav.* **82**, 311-319.

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

Dalesman, S., Karnik, V. and Lukowiak, K. (2011c). Sensory mediation of memory blocking stressors in the pond snail *Lymnaea stagnalis*. *J. Exp. Biol.* **214**, 2528-2533.

Dalesman, S., Sunada, H., Teskey, M. L. and Lukowiak, K. (2013). Combining stressors that individually impede long-term memory blocks all memory processes. *PLoS ONE* **8**, e79561.

Damasio, A. (2010). *Self Comes to Mind: Constructing the Conscious Brain*. New York, NY: Pantheon Books.

De Caigny, P. and Lukowiak, K. (2008). Crowding, an environmental stressor, blocks long-term memory formation in Lymnaea. *J. Exp. Biol.* **211**, 2678-2688.

Diamond, D. M., Campbell, A. M., Park, C. R., Halonen, J. and Zoladz, P. R. (2007). The temporal dynamics model of emotional memory processing: a synthesis on the neurobiological basis of stress-induced amnesia, flashbulb and traumatic memories, and the Yerkes-Dodson law. *Neural Plast.* **2007**, 60803.

Field, D. T., Williams, C. M. and Butler, L. T. (2011). Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions. *Physiol. Behav.* **103**, 255-260.

Francis, S. T., Head, K., Morris, P. G. and MacDonald, I. A. (2006). The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. *J. Cardiovasc. Pharmacol.* **47**, S215-S220.

Fruson, L., Dalesman, S. and Lukowiak, K. (2012). A flavonol present in cocoa [(−)epicatechin] enhances snail memory. *J. Exp. Biol.* **215**, 3566-3576.

Grassi, D., Desideri, G., Necozione, S., Lippi, C., Casale, R., Properzi, G., Blumberg, J. B. and Ferri, C. (2008). Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate. *J. Nutr.* **138**, 1671-1676.

Haney, J. and Lukowiak, K. (2001). Context learning and the effect of context on memory retrieval in *Lymnaea*. *Learn. Mem.* **8**, 35-43.

Hatakeyama, D., Okuta, A., Otsuka, E., Lukowiak, K. and Ito, E. (2014). Consolidation of long-term memory by insulin in *Lymnaea* is not brought about by changing the number of insulin receptors. *Commun. Integr. Biol.* **6**, e23955.

Hebb, D. O. (1955). Drives and the C. N. S. (conceptual nervous system). *Psychol. Rev.* **62**, 243-254.

Heiss, C., Keen, C. L. and Kelm, M. (2010). Flavanols and cardiovascular disease prevention. *Eur. Heart J.* **31**, 2583-2592.

Ito, E., Yamagishi, M., Hatakeyama, D., Watanabe, T., Fujito, Y., Dyakonova, V. and Lukowiak, K. (2015a). Memory block: a consequence of conflict resolution. *J. Exp. Biol.* **218**, 1699-1704.

Ito, E., Yamagishi, M., Sakakibara, M., Fujito, Y. and Lukowiak, K. (2015b). The Yerkes–Dodson law and appropriate stimuli for conditioned taste aversion in *Lymnaea. J. Exp. Biol.* **218**, 336-339.

Karnik, V., Braun, M., Dalesman, S. and Lukowiak, K. (2011). Sensory input from the osphradium modulates the response to memory-enhancing stressors in *Lymnaea stagnalis*. *J. Exp. Biol.* **215**, 536-542.

Knezevic, B. and Lukowiak, K. (2014). The flavonol epicatechin reverses the suppressive effects of a stressor on long-term memory formation. *J. Exp. Biol* **217**, 4004-4009.

Knezevic, B., Dalesman, S., Karnik, V., Byzitter, J. and Lukowiak, K. (2011). Low external environmental calcium levels prevent forgetting in *Lymnaea*. *J. Exp. Biol.* **214**, 2118-2124.

Kojima, S., Sunada, H., Sakakibara, M., Lukowiak, K. and Ito, E. (2015). Function of insulin in snail brain in associative learning. *J. Comp. Physiol. A* **201**, 969-981.

Kuriyama, S., Hozaka, A., Ohmori, K., Shimazu, T., Matsui, T., Ebihara, S., Awata, S., Nagatomi, R., Arai, H. and Tsuji, I. (2006). Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project. *Am. J. Clin. Nutr.* **83**, 355-361.

Letenneur, L., Proust-Lima, C., Le Gouge, A., Dartigues, J. F. and Barberger-Gateau, P. (2007). Flavonoid intake and cognitive decline over a 10-year period. *Am. J. Epidemiol.* **165**, 1364-1371.

Lukowiak, K., Ringseis, E., Spencer, G., Wildering, W. and Syed, N. (1996). Operant conditioning of aerial respiratory behavior in *Lymanea stagnalis*. *J. Exp. Biol.* **199**, 683-691.

Lukowiak, K., Cotter, R., Westly, J., Ringseis, E., Spencer, G. and Syed, N. (1998). Long term memory of an operantly conditioned respiratory behaviour in *Lymnaea stagnalis*. *J. Exp. Biol.* **201**, 877-882.

Lukowiak, K., Adatia, N., Krygier, D. and Syed, N. (2000). Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. *Learn. Mem.* **7**, 140-150.

Lukowiak, K., Fras, M., Smyth, K., Wong, C. and Hittel, K. (2007). Reconsolidation and memory infidelity in *Lymnaea*. *Neurobiol. Learn. Mem.* **87**, 547-560.

Lukowiak, K., Martens, K., Rosenegger, D., Browning, K., de Caigny, P. and Orr, M. (2008). The perception of stress alters adaptive behaviours in *Lymnaea stagnalis*. *J. Exp. Biol.* **211**, 1747-1756.

Lukowiak, K., Orr, M., de Caigny, P., Lukowiak, K. S., Rosenegger, D., Han, J. I. and Dalesman, S. (2010). Ecologically relevant stressors modify long-term memory formation in a model system. *Behav. Brain Res.* **214**, 18-24.

Lukowiak, K., Sunada, H., Teskey, M., Lukowiak, K. and Dalesman, S. (2014). Environmentally relevant stressors alter memory formation in the pond snail *Lymnaea*. *J. Exp. Biol.* **217**, 76-83.

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

Mastroiacovo, D., Kwik-Uribe, C., Grassi, D., Necozione, S., Raffaele, A., Pistacchio, L., Righetti, R., Bocale, R., Lechiara, M. C., Marini, C. et al. (2015). Cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects: the Cocoa, Cognition, and Aging (CoCoA) Study—a randomized controlled trial. *Am. J. Clin. Nutr.* **101**, 538-548.

McComb, C., Rosenegger, D., Varshney, N., Kwok, H.-Y. and Lukowiak, K. (2005). Operant conditioning of an *in vitro* CNS-pneumostome preparation of *Lymnaea*. *Neurobiol. Learn. Mem.* **84**, 9-24.

Milner, B., Squire, L. R. and Kandel, E. R. (1998). Cognitive neuroscience and the study of memory. *Neuron* **20**, 445-468.

Morris, M. C. (2012). Nutritional determinants of cognitive aging and dementia. *Proc. Nutr. Soc.* **71**, 1-13.

Murakami, J., Okada, R., Sadamoto, H., Kobayashi, S., Mita, K., Sakamoto, Y., Yamagishi, Y., Hatakeyama, D., Otsuka, E., Okuta, A. et al. (2013a). Involvement of insulin-like peptide in long-term synaptic plasticity and long-term memory of the pond snail *Lymnaea stagnalis*. *J. Neurosci.* **33**, 371-383.

Murakami, J., Okada, R., Fujito, Y., Sakakibara, M., Lukowiak, K. and Ito, E. (2013b). Paired pulse ratio analysis of insulin-induced synaptic plasticity in the snail brain. *J. Exp. Biol.* **216**, 1771-1773.

Nehlig, A. (2012). The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. *Br. J. Clin. Pharm.* **75**, 716-727.

Nurk, E., Refsum, H., Drevon, C. A., Tell, G. S., Nygaard, H. A., Engedal, K. and Smith, A. D. (2009). Intake of flavonoid-rich wine, tea, and chocolate by elderly men and women is associated with better cognitive test performance. *J. Nutr.* **139**, 120-127.

Orr, M. V., Hittel, K. and Lukowiak, K. (2009). 'Different strokes for different folks': geographically isolated strains of *Lymnaea stagnalis* only respond to sympatric predators and have different memory forming capabilities. *J. Exp. Biol.* **212**, 2237-2247.

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

Parvez, K., Moisseev, V. and Lukowiak, K. (2006). A context-specific single contingent-reinforcing stimulus boosts intermediate-term memory into long-term memory. *Eur. J. Neurosci.* **24**, 606-616.

Rosenegger, D., Wright, C. and Lukowiak, K. (2010). A quantitative proteomic analysis of long-term memory. *Mol. Brain* **3**, 9.

Sangha, S., Scheibenstock, A. and Lukowiak, K. (2003a). Reconsolidation of a long-term memory in *Lymnaea* requires new protein and RNA synthesis and the soma of RPeD1. *J. Neurosci.* **23**, 8034-8040.

Sangha, S., Scheibenstock, A., Morrow, R. and Lukowiak, K. (2003b). Extinction requires new RNA and protein synthesis and the soma of the cell RPeD1 in *Lymnaea stagnalis*. *J. Neurosci.* **23**, 9842-9851.

Sangha, S., Scheibenstock, A., McComb, C. and Lukowiak, K. (2003c). Intermediate and long-term memories of associative learning are differentially affected by transcription *versus* translation blockers in *Lymnaea*. *J. Exp. Biol.* **206**, 1605-1613.

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

Scheibenstock, A., Krygier, D., Haque, Z., Syed, S. and Lukowiak, K. (2002). The soma of RPeD1 must be present for LTM formation of associative learning in *Lymnaea*. *J. Neurophysiol.* **88**, 1584-1591.

Sorond, F. A., Lipsitz, L. A., Hollenberg, N. K. and Fisher, N. (2008). Cerebral blood flow response to flavanol-rich cocoa in healthy elderly humans. *Neuropsychiatr. Dis. Treat.* **4**, 433-440.

Spencer, G., Syed, N. and Lukowiak, K. (1999). Neural changes following operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. *J. Neurosci.* **19**, 1836-1843.

Spencer, G. E., Kazmi, M. H., Syed, N. I. and Lukowiak, K. (2002). Changes in the activity of a CPG neuron after reinforcement of an operantly conditioned behavior in *Lymnaea*. *J. Neurophysiol.* **88**, 1915-1923.

Syed, N. I., Bulloch, A. G. M. and Lukowiak, K. (1990). In vitro reconstruction of the respiratory central pattern generator (CPG) of the mollusk *Lymnaea*. *Science* **250**, 282-285.

Syed, N. I., Ridgway, R. L., Lukowiak, K. and Bulloch, A. G. M. (1992). Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. *Neuron* **8**, 767-774.

van Praag, H., Lucero, M. J., Yeo, G. W., Stecker, K., Heivand, N., Zhao, C., Yip, E., Afanador, M., Schroeter, H., Hammerstone, J. et al. (2007). Plant-derived flavanol (−)epicatechin enhances angiogenesis and retention of spatial memory in mice. *J. Neurosci.* **27**, 5869-5878.

Yerkes, R. M. and Dodson, J. D. (1908). The relation of strength of stimulus to rapidity of habit-formation. *J. Comp. Neurol. Psychol.* **18**, 459-482.