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RESEARCH ARTICLE

The flavonol epicatechin reverses the suppressive effects of a stressor on long-term memory formation

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ABSTRACT

Learning and subsequent memory formation are influenced by both environmental and lifestyle factors, such as stress and diet. Epicatechin, a plant flavonol found in cocoa, red wine and green tea enhances long-term memory (LTM) formation in *Lymnaea*. By contrast, an ecologically relevant stressor, low-calcium pond water, suppresses LTM formation. We tested the hypothesis that epicatechin overcomes the suppressive effects of the stressor on LTM formation in the continued presence of the stressor. Snails trained in low-calcium pond water exhibit learning but not LTM. Epicatechin (15 mg l^{-1}) in control pond water enhances LTM formation. When epicatechin was added to the low-calcium pond water an enhanced LTM similar to that seen in control pond water was observed. Thus, a naturally occurring bioactive plant compound was able to overcome the suppressive effects of an ecologically relevant stressor on LTM formation.

KEY WORDS: Long-term memory formation, *Lymnaea*, Environmental stressors, Epicatechin

INTRODUCTION

As pointed out by Selye (Selye, 1973): 'Everybody knows what stress is and nobody knows what it is'. Here, we define stress as a 'state' that requires a physiological, psychological or behavioural readjustment or modification in order to maintain the well being of the organism. It is well appreciated that stress alters memory formation. This has been known since the time of Bacon (Bacon, 1620) and has been codified in the 'Yerkes–Dodson Law' (Yerkes and Dodson, 1908). We have often referred to this as the Goldilocks rule: too much or too little stress suppresses formation of long-term memory (LTM). In many instances contradictory results (suppressed or enhanced memory) are obtained with a specific stressor (Shors, 2004). Part of the confusion regarding how different stressors alter memory formation is due to the complexity of the brain, the multitude of behaviours tested and the sex of the subject, in addition to the different ways in which stressors act. We have attempted to surmount these difficulties by using a simple model system (aerial respiratory behaviour in *Lymnaea*) in animals with a less-complex brain and a relatively simple tractable behaviour (Lukowiak et al., 1996; Lukowiak et al., 1998; Lukowiak et al., 2000). We have shown that different stressors significantly alter LTM formation and at the same time alters the excitability of a neuron (RPeD1) known to be a necessary locus of LTM formation (Scheibenstock et al., 2002; Sangha et al., 2003a; Sangha et al., 2003b; Sangha et al., 2003c; Sangha et al., 2005; Orr and Lukowiak, 2008; Dalesman et al., 2011a). Therefore, the changes effected in RPeD1 by the various stressors used are consistent with the observed effect of the stressor on LTM formation. Finally, we sought to use only ecologically relevant stressors that *Lymnaea* would encounter in their natural environment (Lukowiak et al., 2010).

Although learning and memory are conceptually and mechanistically linked, they are separate processes each with their own 'rules and regulations' (Milner et al., 1998). In *Lymnaea* LTM, which persists for at least 24 h, is dependent on altered gene activity and new protein synthesis (Sangha et al., 2003b; Parvez et al., 2005). We have found that a wide variety of ecologically relevant stressors alter LTM formation (Lukowiak et al., 2008; Lukowiak et al., 2010). For example, detection of predator smells such as crayfish effluent (CE) enhanced both vigilance behaviours and LTM formation (Orr et al., 2007; Orr et al., 2009; Orr and Lukowiak, 2008); while crowding, low environmental calcium, and certain heavy metals (Zn and Cd) and H_2S in pond water block LTM formation (Rosenegger et al., 2004; De Caigny and Lukowiak, 2008; Dalesman et al., 2011a; Dalesman et al., 2011b; Byzitter et al., 2012). A comprehensive summary of which ecologically relevant stressors significantly alter LTM formation can be found in two recent reviews (Lukowiak et al., 2014). It has also been shown that a flavonoid, epicatechin, found in dark chocolate, green tea and red wine (Kühnau, 1976; Matsuoka et al., 1995; Galliet al., 2002; Youdim et al., 2002) enhances LTM formation in *Lymnaea* (Fruson et al., 2012). However, the enhancement brought about by epicatechin occurs via a different mechanism than that brought about by predator detection (Il-Han et al., 2010; Fruson et al., 2012).

Exposing snails to epicatechin significantly enhanced the ability of snails to form LTM (Fruson et al., 2012). The epicatechin-enhanced LTM formed faster, persisted longer, and was more resistant to extinction. Moreover, epicatechin did not alter other behavioural tests (locomotion, baseline breathing rates, etc.) in *Lymnaea*, thus exemplifying specificity for the drug to interact with the memory-forming neuronal pathways (Fruson et al., 2012). Thus, exposure of *Lymnaea* to epicatechin must somehow alter the ability of neurons to cause new protein synthesis and altered gene activity in neurons essential for LTM formation. Exactly how epicatechin does this at the molecular level is not yet known.

A question that has to be addressed is whether low-calcium pond water is a stressor for *Lymnaea*. First, it is important to know that *Lymnaea* are calciphiles, that is, they obtain their necessary levels of calcium from pond water and not from food sources (Boycott, 1936; Madsen, 1987; Young, 1975). *Lymnaea* normally require >20 mg l^{-1} Ca^{2+} to survive and prosper in natural populations, and are not found in ponds with low levels of dissolved calcium (Van Der Borght and Van Puymbroeck, 1966). Calcium is required for a range of processes in *Lymnaea*, such as basic cellular activity, maintenance of homeostatic calcium blood levels, shell deposition and shell thickening for protection against predators (Rundle et al.,

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2004). Pond water levels containing about 80 mg l^{-1} Ca^{2+} are ideal for thriving populations of *Lymnaea* (Greenaway, 1971). Acute exposure to such low external calcium levels alone is enough to result in a decrease in locomotion and aerial respiration in *Lymnaea* (Dalesman and Lukowiak, 2010). We interpret these findings to indicate that low calcium is a potent stressor for *Lymnaea*.

Only a brief exposure to low calcium conditions (~1 h) is sufficient to impair new LTM formation (Dalesman et al., 2011a). Although the mechanism is not yet fully understood, detection of the low environmental calcium by sensory neurons in the osphradium (Karnik et al., 2012) triggers gene changes in neurons, such as RPeD1, that are incompatible with LTM formation. What these gene changes are in neurons and what role they play in calcium homeostasis are not known. It is known that low calcium levels alter the activity of RPeD1, which is a necessary site of LTM formation. In a low calcium environment a decreased membrane input resistance was observed which did not change with operant conditioning. Furthermore, there was no associated change in the excitability of this neuron following training, as occurs in pond water with normal calcium levels (Dalesman et al., 2011a).

As both the environment and life-style choices (e.g. diet) alter LTM formation we questioned whether a substance such as epicatechin could overcome the negative effects of a stressor that blocks LTM formation in *Lymnaea*. Because snails exposed to low-calcium pond water (20 mg l^{-1}) are not able to make new LTM (Dalesman et al., 2011a; Knezevic et al., 2011), would the addition of epicatechin to the low-calcium pond water reverse or mitigate the effects that low calcium has on memory? This is an important question to answer because it is unclear whether in the continued presence of a stressor, the effects of the stressor can be overcome by life-style choices such as diet.

RESULTS

A naive cohort of snails maintained in a low-calcium environment (20 mg l^{-1}) was trained (two 0.5 h sessions (TS1 and TS2) with a 1 h interval between sessions (Fig. 1). This training procedure would normally result in LTM formation. However, because the snails were trained and maintained throughout the course of the experiment in a low-calcium environment, LTM was not seen. A repeated-measures analysis of variance (ANOVA) indicated that there was a significant difference in mean attempted pneumostome openings between the three sessions ($F_{2,36}$=26.35, P=0.001). A *post hoc* paired-sample *t*-test was used for all multiple comparisons among group means. There was a significant decrease in attempted pneumostome openings from training session 1 (14.21±5.44; mean ± s.d.) to training session 2 (8.68±4.14; t_{18}=3.65, P=0.002). This indicates that learning occurred and intermediate term memory (ITM) formed. However, LTM was not formed because the number of attempted pneumostome openings in the memory test session was not significantly less than training session 1 and was significantly greater than training session 2. In fact, results of the memory test session were significantly greater than for training session 1 (19.84±5.18; t_{18}=–3.55, P=0.002).

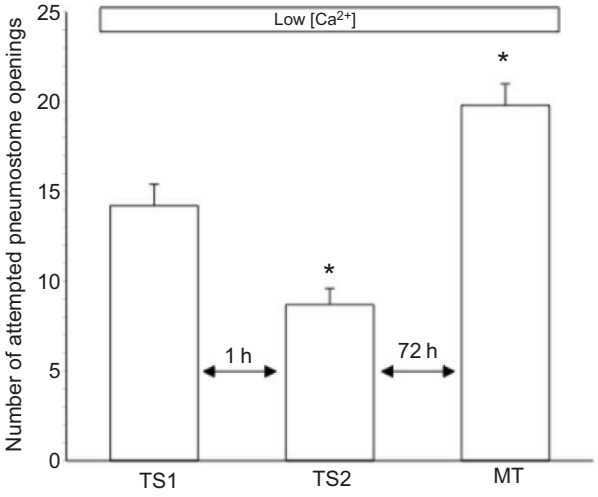

Fig. 1. Long-term memory formation does not occur in *Lymnaea stagnalis* pond snails in low-calcium pond water. Results are means ± s.e.m. from two 0.5 h training sessions (TS1 and TS2) separated by 1 h, and a 0.5 h memory test session (MT) carried out 72 h later. Snails (n=19) were kept in low calcium (20 mg l^{-1}) throughout the experiment. *P<0.001.

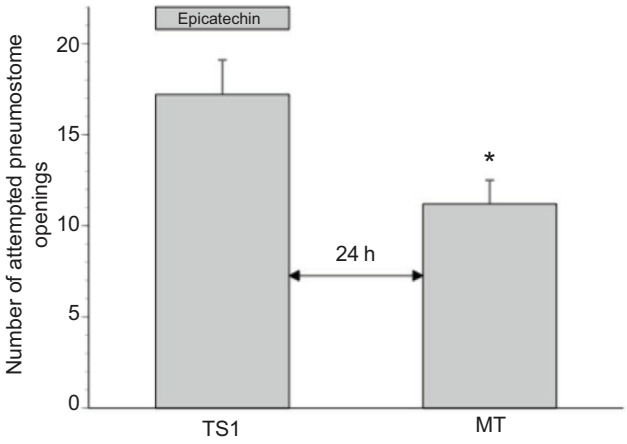

Fig. 2. Epicatechin enhances LTM formation. Results are means ± s.e.m. for snails (n=21) during a single 0.5 h training session and a 0.5 h memory test session 24 h later. Snails were kept in regular pond water (80 mg l^{-1} Ca^{2+}) throughout the experiment, except for during training when the water was also treated with 15 mg l^{-1} epicatechin. *P<0.001 compared with TS1.

We next tested whether training snails in the presence of epicatechin in normal calcium pond water (80 mg l^{-1}) caused enhanced LTM formation (Fig. 2). Typically a single 0.5 h training session does not result in LTM. Following the single training session in 15 mg l^{-1} epicatechin supplemented pond water memory was observed 24 h later (calcium levels kept at 80 mg l^{-1} throughout experiment). A paired-sample *t*-test showed that there was a significant difference in the number of attempted openings between the two sessions. More specifically, the number of attempted pneumostome openings were significantly lower in the memory test session (11.19±6.17) than in the training session (17.24±8.69; t_{20}=3.45, P=0.003). Thus, epicatechin enhanced LTM formation because now a single 0.5 h training sessions was sufficient to produce LTM.

Having shown here that epicatechin enhanced the ability of *Lymnaea* to form LTM following a single training session, we wanted to determine how long the epicatechin-induced LTM persisted. In normal pond water (i.e. without epicatechin), two 0.5 h training sessions with a 1 h interval between sessions results in LTM lasting 24 h, but not 48 h. We thus trained snails using this training procedure in epicatechin augmented normal calcium pond water and tested for LTM 72 h later (Fig. 3). A repeated-measures analysis of variance (ANOVA) indicated that there was a significant difference in the number of attempted pneumostome openings between the three sessions ($F_{2,28}$=8.21, P=0.002). There was a significant decrease in the number of attempted pneumostome openings from

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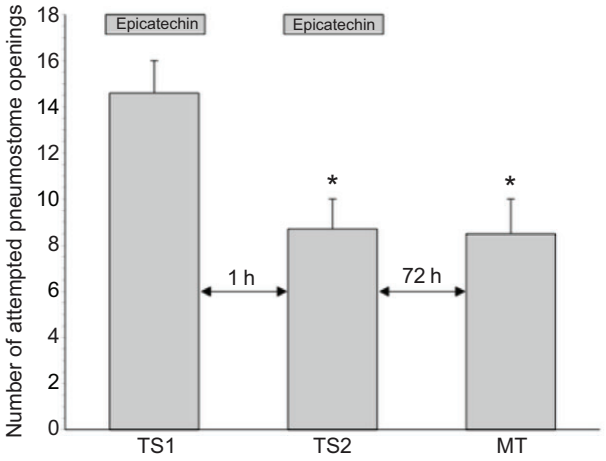

Fig. 3. Epicatechin enhances LTM formation with two training sessions. Mean ± s.e.m. pneumostome openings for snails (n=15) during two 0.5 h training sessions (TS1 and TS2) separated by 1 h, and a 0.5 h memory test session 72 h later. Snails were kept in regular pond water (80 mg l^{-1} Ca^{2+}) throughout the experiment, except during the two training sessions when water was also treated with 15 mg l^{-1} epicatechin. *P<0.001 compared with TS1.

training session 1 (14.60±5.44) to training session 2 (8.73±4.88; t_{14}=3.99, P=0.001), as well as from training session 1 to the 72 h memory test (8.47±5.80; t_{14}=3.35, P=0.005). Thus, enhanced LTM formation was observed 72 h after training.

We next examined whether epicatechin mitigated or reversed the ability of the low-calcium environment to block LTM formation. Thus, we trained snails in epicatechin or low calcium pond water (20 mg l^{-1}) and tested for memory 24 h later (Fig. 4). Snails were maintained through out the experiment in the low calcium pond water. An ANOVA indicated that there was a significant difference in the number of attempted pneumostome openings between the three sessions ($F_{2,36}$=9.03, P=0.001). We found that there was a significant decrease in pneumostome openings from training session 1 (15.63±5.71) to training session 2 (11.11±3.64; t_{18}=3.06, P=0.007), as well as from training session 1 to the memory test 24 h later (10.32±4.72; t_{18}=4.14, P=0.001). Thus, low-calcium pond water no longer blocked LTM formation in the presence of epicatechin.

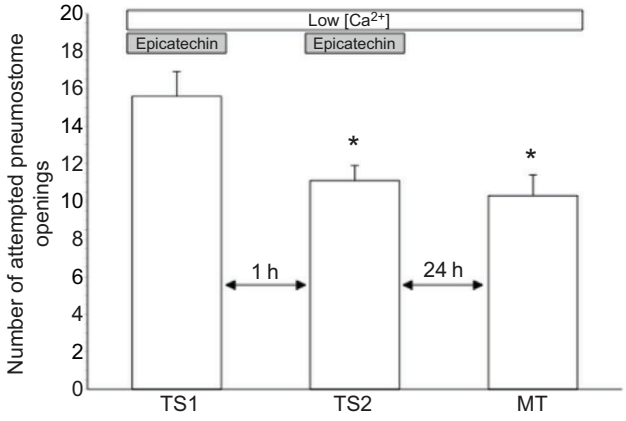

Fig. 4. Epicatechin overcomes the suppressive effect of low calcium on LTM formation. Mean ± s.e.m. pneumostome openings for snails (n=19) during two 0.5 h training sessions (TS1 and TS2) separated by 1 h, and a 0.5 h memory test session 24 h later. Snails were kept in low calcium (20 mg l^{-1}) water throughout the experiment, and 15 mg l^{-1} epicatechin was added during TS1 and TS2. *P<0.001 compared with TS1.

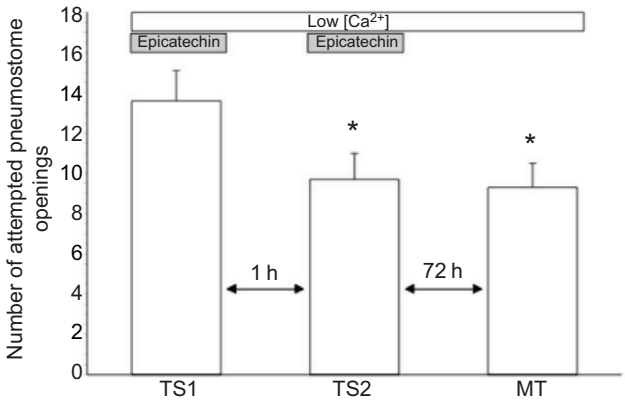

Fig. 5. Epicatechin continues to cause LTM enhancement even in low-calcium pond water. Mean ± s.e.m. pneumostome openings for snails (n=19) during two 0.5 h training sessions (TS1 and TS2) separated by 1 h, and a 0.5 h memory test session 72 h later. Snails were kept in low-calcium (20 mg l^{-1}) water throughout the experiment, and 15 mg l^{-1} epicatechin was added during TS1 and TS2. *P<0.001 compared with TS1.

As a final test of the ability of epicatechin to enhance LTM formation when snails were maintained in the low calcium environment we asked whether epicatechin would still be able to cause an LTM persisting for 72 h (Fig. 5). An ANOVA indicated that there was a significant difference in the number of attempted pneumostome openings between the three sessions ($F_{2,36}$=4.07, P=0.026). There was a significant decrease in pneumostome openings between training session 1 (13.63±6.37) and training session 2 (9.68±5.59; t_{18}=2.14, P=0.046), as well as from training session 1 to the 72 h memory test (9.32±5.37; t_{18}=2.23, P=0.039). Thus, epicatechin in the low-calcium pond water continues to enhance LTM formation that persists for at least 72 h. Epicatechin therefore has the ability to overcome the suppressive effects of this stressor on LTM formation.

DISCUSSION

The data we present here demonstrate that the suppressive effects of a stressor (low environmental calcium) on LTM formation is reversed by a flavonol (epicatechin). We believe that this is a significant finding because it shows that a food product has the ability to counteract the effects brought about by an ecologically relevant stressor on LTM formation. Epicatechin, which is present in dark chocolate, has been thought to improve various aspects of cognition in rodents and humans, with some reports suggesting that it has positive effects on mood (i.e. anxiolytic), which may be why chocolate is often consumed under emotional distress (Yamada et al., 2009; Nurk et al., 2009; Messaoudi et al., 2008; Parker et al., 2006; Nehlig, 2013). To our knowledge, however, the ability of epicatechin to overcome the negative cognitive effects of stress has not previously been demonstrated in a model system used to elucidate the causal mechanisms of learning and memory formation.

We defined stress here as a condition that significantly alters the physiological or psychological homeostasis of an organism (Lukowiak et al., 2014). The idea that exposure to a low-calcium environment, even for a short period of time (e.g. 1 h), is stressful for *Lymnaea* is based on a number of behavioural and physiological observations. This species of snail is a calciphile, meaning it has to absorb most of its calcium requirements directly from its pond water. *Lymnaea* are only found in ponds where the environmental calcium is greater than 20 mg l^{-1} (Boycott, 1936; Young, 1975). When snails are maintained below this level, growth and survival

are reduced (McKillop and Harrison, 1972). An adequate supply of calcium is also needed during reproduction to provision embryos so that they develop properly (Ebanks et al., 2010). A low-calcium environment also results in shell thinning, potentially making them easier prey (Lewis and Magnuson, 1999; Zalizniak et al., 2009). Importantly, in low-calcium pond water, the basic metabolic rate is significantly increased and activity levels, for example locomotion and aerial respiration, are decreased (Dalesman and Lukowiak, 2010). These findings should not be too surprising, because calcium uptake from the environment requires energy (i.e. 'pumps') when external calcium is below ~50 mg l^{-1} (Greenaway, 1971). Finally, even following short (~1 h) periods of exposure to low levels of calcium (20 mg l^{-1}), *Lymnaea* no longer formed LTM, although they still demonstrated learning and ITM (Dalesman et al., 2011a). Finally, given the opportunity, *Lymnaea* orientate toward a calcium-rich environment over a calcium-poor environment (Piggott and Dussart, 1995). Thus, we are satisfied that exposing snails to a low calcium environment is perceived by the snail as a stressful situation.

As shown both previously and here, snails did not form LTM in a low environmental calcium situation. What had not been tested previously was whether snails would adapt over the course of a few days to the low-calcium environment following operant conditioning. That is, it was possible that with longer exposure times following learning to the low calcium environment snails would exhibit LTM. However, when we tested this here, snails maintained in low calcium throughout the duration of the experiment did not show LTM after two training sessions and a memory test session 72 h later. However, we did find an interesting and unexpected result from this experiment. The number of attempted pneumostome openings in the memory test session was significantly greater than the number in the initial training session (i.e. TS1). That is, snails appeared to be in a sensitized state and attempted significantly more pneumostome openings, even though they were only in hypoxic conditions for a relatively brief time. It may be possible that having low calcium levels for such a long duration is simply too great of a stressor on snails, forcing them to hyper-activate all survival instincts, one of which is aerial respiration.

We had also previously demonstrated the ability of epicatechin to enhance LTM formation, but those experiments were all performed on naive, unstressed snails (Fruson et al., 2012). We initially hypothesized, wrongly as it turns out, that epicatechin would at best just counterbalance the effects caused by the low-calcium environment. We arrived at that hypothesis based on earlier experiments with the simultaneous presentation of two different stressors that significantly alter LTM formation in *Lymnaea.* The two stressors used were: (1) low calcium, which as we have seen, suppresses LTM and (2) predator detection, which enhances LTM formation (Dalesman and Lukowiak, 2012; Dalesman et al., 2011a; Dalesman et al., 2011c; Knezevic et al., 2011; Orr and Lukowiak, 2008). We found that operantly trained animals (two 0.5 h training sessions) that were held in the low-calcium environment in the presence of CE, were able to form LTM lasting 24 h (Dalesman and Lukowiak, 2011a) suggesting that training in CE may prevent the memory-blocking effects of a low-calcium environment. However, in control conditions (i.e. no stressors), with two 0.5 h training sessions an LTM persisting for 24 h is normally seen. However, when snails are trained in CE with this procedure LTM persists for 8 days (Orr and Lukowiak, 2008). To confirm whether CE was preventing the effects of low calcium on memory, we tested for LTM 72 h following training in CE. The snails exposed to both the low calcium and CE stressors did not exhibit a 72 h LTM (Dalesman and Lukowiak, 2011b). Thus, in the presence of both stressors, the snails show an identical memory phenotype to the one they show under control conditions (i.e. no stressors present). It appears that the effects of each stressor have effectively canceled each other out. Likewise, when a combination of two stressors (crowding and low calcium, respectively), each of which blocks LTM formation but not learning and ITM formation, was applied to *Lymnaea*, this combination of stressors blocked all memory processes (i.e. learning, short-term, intermediate-term and long-term memory; Dalesman et al., 2013). Thus, it appears that the effects of the stressors were additive. We were therefore surprised that epicatechin in the low-calcium environment enhanced LTM formation as it did in naive snails in normal (i.e. high-calcium) pond water.

Since the time of Bacon in the mid-17th century, researchers have noted that stress can prevent memory formation, but have not, for the most part, been able to effectively mitigate the stressor's effect on LTM formation. Typically, the strategy used is to attempt to alleviate the stressor, but in most instances this is not an effective strategy. Here, we show that it is possible, even when the stressor is still present, to overcome the negative effects of the stressor on LTM formation. We accomplished this using a plant-derived flavonoid, epicatechin. We have previously shown that epicatechin on its own is a potent enhancer of LTM formation. Training snails in the presence of the flavonoid results in a memory that forms faster, persists longer and is more resistant to extinction (Fruson et al., 2012). Here, we show that epicatechin is just as effective in enhancing LTM formation in the continued presence of the stressor. Epicatechin does not remove the stressor, rather it overcomes the negative effects of the stressor on LTM formation. This may be considered analogous to the anxiolytic effect that dark chocolate has according to some reports in humans (Nehlig, 2013).

It is unclear what the neuronal mechanism(s) of epicatechin-induced enhancement of LTM is. Our preliminary unpublished data show that it does not cause changes in the activity and excitability of RPeD1 that CE does (Orr and Lukowiak, 2008). Some studies suggest that flavonoids 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 ITM and LTM formation in *Lymnaea* (Rosenegger et al., 2010; Rosenegger and Lukowiak, 2010). Therefore, alteration of protein kinase activity by epicatechin could be the mechanism by which memory formation is enhanced in *Lymnaea*. However, the mechanism of this relationship between epicatechin and LTM is still unknown. In mammalian preparations it has been suggested that the antioxidant properties of epicatechin, which prevent neuronal cells from oxidative stress, are the basis for enhanced cognitive seen with epicatechin (Hanasaki et al., 1994). However, since the effects of epicatechin shown here occur within minutes of testing, we do not believe epicatechin works via this mechanism. Finally, we know that although CE, which also enhances LTM formation, works via a serotonergic pathway (Il-Han et al., 2010), blocking this pathway using mianserin (a serotonin receptor antagonist) does not effect the same LTM enhancement induced by epicatechin (Fruson et al., 2012).

In *Lymnaea*, stressors that alter LTM formation come in many forms (temperature spikes, resource restriction, crowding, and predator detection) and the complex interactions of these elements often yield unpredictable results (Lukowiak et al., 2014). Because epicatechin was able to alleviate the detrimental effects of stress on LTM formation, this finding may have important implications for

humans. This finding indicates that through ingesting foods rich in flavonoids, we may be able to boost memory retention during stressful conditions, when memory recall might be most beneficial. These results, therefore, provide the basis for future studies in *Lymnaea* in order to elucidate how dietary substances such as the flavonoids cause changes in neurons, which play a necessary role in memory formation in the presence of different stressors that affect memory.

MATERIALS AND METHODS

Animals

Adult pond snails (*Lymnaea stagnalis* Linnaeus 1758) 25±1 mm spire height, originally obtained from a stock from Vrije Universeit, Amsterdam were used. These snails were derived from snails collected from ditches in polders located near Utrecht in the 1950s. The adult snails used here were raised in aquaria with artificial pond water (with a Ca^{2+} concentration of 80±5 mg l^{-1}) at the University of Calgary. The artificial pond water was made using deionized water with 0.26 g l^{-1} Instant Ocean (Spectrum Brands Inc., Madison, WI, USA) and calcium sulphate dihydrate added so that the final calcium concentrations were raised to 80 mg l^{-1} Ca^{2+}. Low-calcium water was made in a similar manner with less calcium sulphate dihydrate added so that final calcium concentration was 20 mg l^{-1} (see Dalesman and Lukowiak, 2010 for more details). Tanks were maintained at a temperature of 20±1°C, and snails were housed in a 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 were altered, as discussed below. All other conditions (temperature, density, food) remained unchanged.

Training procedure

Operant conditioning training sessions (TS, 0.5 h) and memory testing (MT, 0.5 h) sessions were conducted in hypoxic pond water. Water was made hypoxic by bubbling with nitrogen for 20 min prior to transferring the snails. This was carried out to increase average baseline pneumostome openings. The periods of rest between training sessions (1 h) as well as leading up to the memory testing sessions (24 or 72 h) were conducted in normally oxygenated artificial pond water at the specified calcium concentration (normal and low calcium) for the specific experiment. 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.

Snail group conditions

We used five different cohorts of naive snails for the studies described above.

(1) The first group of naive snails (n=19; Fig. 1) was trained (i.e. operantly conditioned) and then tested for LTM in low-calcium pond water (20 mg l^{-1} Ca^{2+}). Following the first training session, they were given a 1 h break before being trained a second time. The snails were then transferred to a small, oxygenated tank for 72 h, after which they were tested for 30 min in the testing beaker. Snails were maintained in low-calcium pond water throughout this procedure.

(2) The second group of naive snails (n=21; Fig. 2) was trained (i.e. operantly conditioned) and then tested for LTM in standard pond water (80 mg l^{-1} Ca^{2+}) that was supplemented with 15 mg l^{-1} epicatechin (Sigma-Aldrich, St Louis, MO, USA). They received a single 0.5 h training session and then were tested for LTM 24 h later. They were maintained in normal pond water during the 24 h interval between training and memory testing. They were tested for LTM in pond water without epicatechin. Standard calcium concentrations were maintained throughout the entirety of the procedure.

(3) The third group of snails (n=15; Fig. 3) was trained (i.e. operantly conditioned) and then tested for LTM in standard pond water (80 mg l^{-1} Ca^{2+}) that was supplemented with 15 mg l^{-1} epicatechin (Sigma-Aldrich). They received two 0.5 h training sessions separated by a 1 h interval. LTM was tested 72 h after TS2. They were maintained in normal pond water both during the 1 h interval between TS1 and TS2 and the 72 h interval between training and memory testing. They were tested for LTM in pond water without epicatechin. Standard calcium concentrations were maintained throughout the procedure.

(4) The fourth group of snails (n=19; Fig. 4) was trained (i.e. operantly conditioned) and then tested for LTM in low-calcium pond water (20 mg l^{-1} Ca^{2+}). They received two 0.5 h training sessions separated by a 1 h interval. LTM was tested 24 h after TS2. However, the low-calcium pond water was supplemented with 15 mg l^{-1} epicatechin in the two training sessions. In the memory test session 24 h after TS2 the low-calcium pond water was not supplemented with epicatechin. Low calcium concentrations were maintained throughout the entirety of the procedure.

(5) Lastly, the fifth group of snails (n=19; Fig. 5) was treated in the exact same manner as Cohort 4 snails with the exception that LTM was tested 72 h after TS2. Again, low calcium concentrations were maintained throughout the procedure.

Statistical analyses

Data for each cohort were analysed separately in SPSS Statistics Version 20 (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 lay. In the experiment (Fig. 2) with epicatechin where only a single 0.5 h training session and a single memory test 24 h later was used a paired *t*-test was used to determine whether memory formed.

Competing interests

The authors declare no competing financial interests.

Author contributions

K.L. and B.K. contributed to the conceptual design of experiments, interpretation of findings, execution of some of the experiments, and drafting and revising the article.

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