© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb238055. doi:10.1242/jeb.238055

SHORT COMMUNICATION

# Epicatechin increases the persistence of long-term memory formed by conditioned taste aversion in *Lymnaea*

**Ayaka Itoh[1], Yoshimasa Komatsuzaki[2], Ken Lukowiak[3] and Minoru Saito[1,*]**

## ABSTRACT

We examined the effects of epicatechin (Epi), a flavonoid abundant in green tea and cocoa, on long-term memory (LTM) formed following conditioned taste aversion (CTA) training in *Lymnaea stagnalis*. In CTA training, the snails learnt to avoid a food that initially they liked (i.e. sucrose). Twenty-four hours after CTA training, 67% of the trained snails showed a significant decrease in the feeding behaviour elicited by sucrose. Placing snails in the Epi solution in CTA training did not alter the percentage of snails exhibiting LTM, but it significantly increased LTM persistence. We also examined changes following Epi exposure in spontaneous activity of the cerebral giant cells (CGCs) that modulate feeding behaviour and are necessary for CTA-LTM. Our data suggest that Epi causes a decrease in CGC activity and increases LTM persistence, possibly via a GABAergic mechanism.

**KEY WORDS: Learning and memory, Flavonoid, Cerebral giant cell, γ-Aminobutyric acid, Pond snail**

## INTRODUCTION

Gastropod molluscs are excellent as model systems to understand the causal neuronal mechanisms underlying learning and memory. This is in part due to their relatively simple central nervous system (CNS), containing large identifiable neurons that mediate simple, tractable behaviours. There is a long, successful history of using molluscs such as *Aplysia*, *Limax*, *Hermissenda* (Bailey and Kandel, 1993; Gelperin et al., 1996; Abel and Kandel, 1998; Matzel et al., 1998; Ito et al., 1999; Kimura et al., 1999) and *Lymnaea* (Lukowiak et al., 1996, 1998, 2003; Spenser et al., 1999; Scheibenstock et al., 2002) in the study of how learning and memory are mediated at the neuron level. In addition to the aforementioned studies on classical and operant conditioning, configural learning has also recently been shown in *Lymnaea stagnalis* (Swinton et al., 2019). Here, we focused our attention on conditioned taste aversion (CTA), which is a form of classical conditioning in which the snails learn to avoid a food (e.g. sucrose) that initially they were drawn to (Yamanaka et al., 1999; Sugai et al., 2007).

Recently, flavonoids, which are a group of phytochemicals found in plants, have been shown to enhance cognition in a variety of species including invertebrates (van Praag et al., 2007; Li et al., 2009; Fruson, et al., 2012; Knezevic and Lukowiak, 2014; Swinton et al., 2018). Epicatechin (Epi) is one such flavonoid and is abundant in green tea and cocoa. In *L. stagnalis*, Epi enhanced long-term memory (LTM) formation of an associative memory following operant conditioning of aerial respiration (Fruson et al., 2012). In addition, the application of Epi to *L. stagnalis* immediately reversed the obstruction of learning and memory formation induced by a combination of stressors (Knezevic and Lukowiak, 2014). Here, we report the effects of Epi on LTM formed by CTA in *L. stagnalis*. In this aversive conditioning paradigm, an appetitive stimulus (sucrose) serves as the conditioned stimulus (CS). The CS, which increases the feeding behaviour, is paired with an aversive stimulus (KCl), the unconditioned stimulus (UCS), which inhibits the feeding behaviour. Following successful CTA training, the CS no longer elicits feeding (Ito et al., 2015).

An identified spontaneously active pair of neurons, the cerebral giant cells (CGCs), has been shown to both modulate the neuronal network underling the feeding behaviour (Yeoman et al., 1994a,b; Benjamin, 2012) and be necessary for LTM and its retrieval following single-trial appetitive training (Kemenes et al., 2006) or CTA training (Sunada et al., 2017). The most significant CGC synaptic connections are with the neuron 1 medial (N1M) cell, an interneuron in the central pattern generator (CPG) that co-ordinates rhythmic feeding movements (Yeoman et al., 1996; Kojima et al., 1997, 2001; Ito et al., 2012). In the present study, in addition to studying Epi's effect on CTA-LTM, we also examined the changes in spontaneous activity recorded in the CGC following Epi application and CTA training.

## MATERIALS AND METHODS

### Animals

The *Lymnaea stagnalis* (Linnaeus 1758) used in our experiments were supplied by Prof. Etsuro Ito of Waseda University, Japan. These snails were originally derived from stocks maintained by Vrije University in Amsterdam. All snails were maintained in dechlorinated tap water under a 12 h:12 h light:dark cycle at 20°C and fed *ad libitum* on *Brassica rapa var. peruviridis* (a relative of Bok choy), known as Komatsuna in Japan.

### CTA training

Adult snails randomly chosen were food deprived for 24 h before being subjected to CTA training. Each snail was then moved into a polystyrene Petri dish (90 mm diameter) and immersed in distilled water for 30 min to allow them to acclimate to their new environment. Snails were then immersed in an appetitive solution (10 mmol $l^{-1}$ sucrose) for 15 s. Then, the sucrose solution was quickly removed with an aspirator and was replaced with distilled water, and the feeding response (i.e. number of bites) was measured in distilled water for 5 min (pre-test).

Ten minutes after the pre-test, CTA training was performed. In CTA training, snails were immersed for 15 s in 10 mmol $l^{-1}$ sucrose

[1]Department of Correlative Study in Physics and Chemistry, Graduate School of Integrated Basic Sciences, Nihon University, 3-25-40 Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan. [2]Department of Physics, College of Science and Technology, Nihon University, 1-8-14 Kandasurugadai, Chiyoda-ku, Tokyo 101-8308, Japan. [3]Department of Physiology and Pharmacology, Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1.

*Author for correspondence (msaito@chs.nihon-u.ac.jp)

M.S., 0000-0002-0249-7773

**Received 22 September 2020; Accepted 15 December 2020**

Journal of Experimental Biology (2021) 224, jeb238055. doi:10.1242/jeb.238055

solution (i.e. the CS), and then the sucrose solution was quickly removed with an aspirator, immediately followed by immersion for 15 s in 10 mmol $l^{-1}$ KCl solution (i.e. the UCS). The UCS inhibits the feeding response. Following the presentation of the UCS, snails were immersed either in distilled water (control) or Epi solution (15 mg $l^{-1}$) for 9.5 min. This procedure was repeated 5 times. The concentration of sucrose or KCl solution was determined according to a previous report (Kojima et al., 1996). The concentration of Epi (15 mg $l^{-1}$) was chosen to be equivalent to that for humans. Moreover, at that concentration, Epi does not alter important homeostatic behaviours in *L. stagnalis* (Fruson et al., 2012).

After CTA training, snails were kept in distilled water for 24, 48 or 72 h and then the post-test was performed, which was exactly the same as the pre-test. In each 24 h interval between two post-tests, snails were allowed to feed *ad libitum* only for the first 2 h in order to maintain their motivation for feeding. By comparing the number of bites in the pre-test with that in the 24 h post-test, we determined whether the snail was a 'good' learner or a 'poor' learner (see below).

### Intracellular recording of CGC activity

Snails were anaesthetized in 25% Listerine® before dissection. The CNS was isolated from the body and the CGC was viewed with a microscope. Then, intracellular recordings of the CGC were made using a glass microelectrode filled with 2 mol $l^{-1}$ potassium acetate. *Lymnaea* saline [composition in mmol $l^{-1}$: 24 NaCl, 2 KCl, 2 $MgCl_2$, 4 $CaCl_2$, 0.3 D-glucose, 0.1 $NaH_2PO_4$ and 35.4 Hepes-NaOH (pH 7.9)] was used as the external medium. Signals were amplified with an intracellular recording amplifier (IR-283, NeuroData) and transferred through an AD converter (PowerLab, ADInstruments, Bella Vista, NSW, Australia) to a PC with 1 kHz sampling.

In this way, the change in spontaneous activity of the CGC following Epi application and CTA training was recorded. The concentration of Epi applied was the same as that (15 mg $l^{-1}$) in the behavioural experiment, for the following reasons: (1) 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); (2) in *Lymnaea*, water-soluble substances, such as Epi, can easily cross the skin membrane (Wertz, 2013); (3) snails possess an open circulatory system that allows for direct contact of bio-active substances with the CNS.

### Statistical analysis

All data are expressed as means±s.e.m. For the behavioural experiments, a 2-way ANOVA followed by Tukey's *post hoc* test was used in order to compare the post-test scores between two groups (control versus Epi) at 24, 48 and 72 h, respectively, after CTA training. We compared the mean number of bites in pre- and post-tests in each group. For the intracellular recordings of the CGC, Tukey's *post hoc* test was performed for multiple comparisons when a repeated measures one-way ANOVA tests yielded $P<0.05$. Statistical analysis was performed using GraphPad Prism 7.

## RESULTS AND DISCUSSION

Fig. 1A shows a histogram of the decreasing rate of the feeding response in the 24 h post-test (i.e. 24 h after training). The decreasing rate was measured for 26 snails trained without Epi and 22 snails trained with Epi, and the data from all snails ($n$=48) are combined in the histogram. As shown in Fig. 1A, from their responses, the snails can roughly be divided into two groups: 'good' and 'poor' learners. Good learners are defined as snails that show at least a 40% decrease in the number of bites in the 24 h post-test compared with their pre-test scores. Thus, poor learners are snails whose post-test scores decrease by less than 40%. In the data presented here, 32 of the 48 snails (i.e. 67%) were classified as good learners. In detail, 19 of 26 snails in the control group (without Epi) exhibited the good learning phenotype. In the Epi group, 13 of 22 snails exhibited the good learning phenotype. The percentage of good learners was not significantly different between these two groups ($P$=0.3057, tested by a Chi squared test).

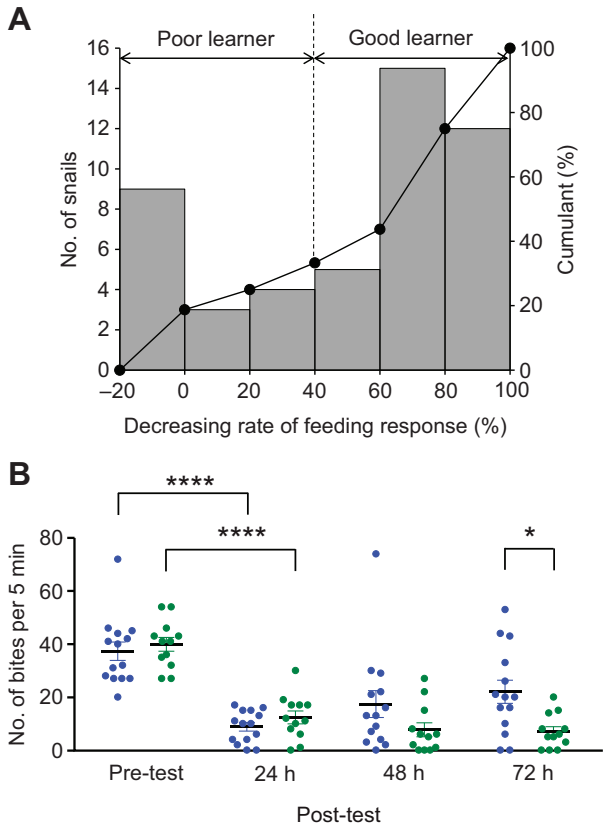


**Fig. 1. Change in feeding response after conditioned taste aversion (CTA) training.** (A) Histogram showing the decreasing rate of the feeding response at the 24 h post-test, as measured for 26 snails trained without epicatechin (Epi) and 22 snails trained with Epi (combined data are shown, $n$=48 total). The cumulant (the ratio of the cumulative number of snails at each rate, from low to high, out of the total number, $n$=48; black circles) is also shown for reference. Snails that showed at least a 40% decrease in the number of bites were defined as good learners, while poor learners were defined as snails whose post-test scores decreased by less than 40%. (B) Feeding responses in the pre- and post-tests for good learners trained without Epi (control, blue circles, $n$=14) and with Epi (green circles, $n$=12). ****$P$<0.0001, *$P$<0.05.

For the good learner phenotype, we performed a 2-way ANOVA followed by Tukey's *post hoc* test on the data presented in Fig. 1B ($n$=14 control, $n$=12 Epi). Here, data for good learners that died after the 24 h post-test were removed from the statistical analysis. In both groups, snails showed a significant decrease in the number of bites in the 24 h post-test (control 37.3±3.5 to 8.8±1.6 bites per 5 min, $P$<0.0001; Epi group 39.9±2.6 to 12.3±2.4 bites per 5 min, $P$<0.0001). Thus, placing snails in the Epi solution in CTA training did not alter their 24 h memory performance.

We next asked whether Epi exposure would affect the persistence of LTM in the good learning phenotype. That is, we compared the post-test scores between two groups (control versus Epi) at 24, 48 and 72 h after CTA training. There was an interaction ($F_{3,96}$=3.776;

Journal of Experimental Biology (2021) 224, jeb238055. doi:10.1242/jeb.238055

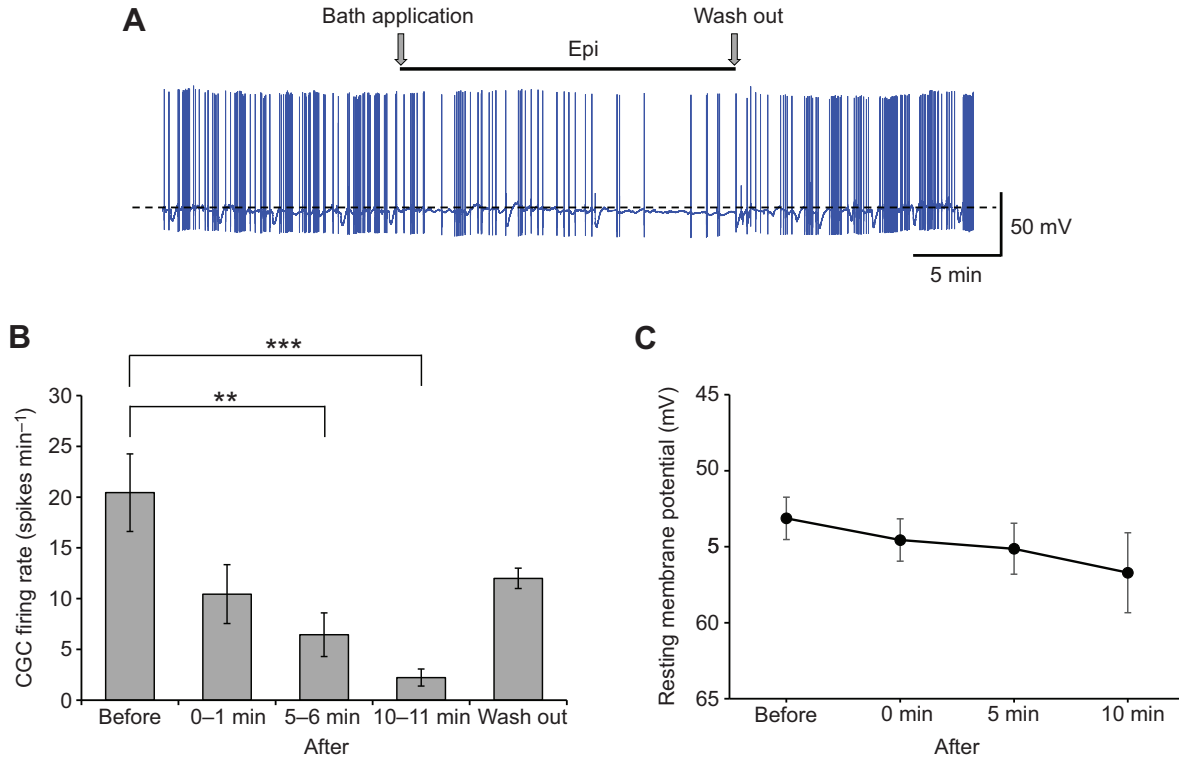


**Fig. 2. Effect of Epi on cerebral giant cell (CGC) activity.** (A) Illustrative data showing the effect of perfusing the CNS of a naive snail with Epi solution (15 mg $l^{-1}$; horizontal bar). The dashed line shows the resting membrane potential before Epi application. (B) CGC firing rate before and after application of Epi ($n$=9), and 30 min after wash out ($n$=3). **$P$<0.01, ***$P$<0.001. (C) CGC resting membrane potential before and after Epi application ($n$=8 for '10 min', $n$=9 for the others).

$P$=0.0131) between the variables, i.e. pre- and post-tests (24, 48 and 72 h) and treatment (control and Epi). When we performed a Tukey's *post hoc* test, we found that there was a significant difference in the memory scores at 72 h between the two groups (control versus Epi, $P$=0.0405) while there was no significant difference in the 24 h ($P$=0.9951) and 48 h ($P$=0.4535) post-tests. Thus, exposing snails to Epi solution resulted in significantly longer memory persistence.

We hypothesized that a possible mechanism underlying the significant effect of Epi on LTM persistence involved an alteration in CGC activity. We directly tested this by perfusing the CNS of naive snails with Epi solution (15 mg $l^{-1}$). An example of the results is shown in Fig. 2A, while quantitative data on firing rate are presented in Fig. 2B. As can be seen, Epi caused an immediate and significant decrease in the spontaneous activity of CGCs. A one-way ANOVA ($F_{4,34}$=7.23; $P$=0.0003) showed $P$=0.0047 at 5 min or $P$=0.0002 at 10 min after Epi application compared with the spontaneous activity before application. The spontaneous activity did not completely recover to the level before Epi application at 30 min after wash out. However, there was a tendency to gradually recover, and therefore we confirmed that the decrease in the spontaneous activity of the CGCs is due to the effect of Epi. Additionally, this effect was accompanied by a trend towards hyperpolarization of the resting membrane potential of the CGCs (Fig. 2C).

We then examined the spontaneous activity of CGCs following CTA training (without Epi). Fig. 3A shows illustrative data from CGCs from a naive snail, a poor learner and a good learner, 24 h after training. In Fig. 3B, we plot the firing rate of the CGCs for naive snails ($n$=4), poor learners ($n$=3) and good learners ($n$=5). A one-way ANOVA ($F_{2,9}$=7.342; $P$=0.0129) showed that the firing rate was significantly lower in the good learners ($P$=0.0106) than in naive snails. This result is consistent with a previous study showing that a slower rate of CGC firing leads to a slower rate of CPG activity that drives feeding behaviour (Yeoman et al., 1996), and supports another recent study showing that the CGCs are necessary for the formation and recall of memory of CTA (Sunada et al., 2017). It should be noted that there was no significant difference between the firing rates of poor and good learners ($P$=0.1647), suggesting that the CGCs maintained the memory trace even in the poor learners but the expression of the memory was suppressed by some other mechanism.

It has previously been reported that γ-aminobutyric acid (GABA) is involved in some behaviours of *L. stagnalis* (Romanava et al., 1996; Mocca et al., 2009), and therefore we hypothesized that CGC firing is regulated by GABAergic neurons. This hypothesis was supported by our preliminary data showing that a GABA antagonist, bicuculline, increased the spontaneous activity of the CGC (data not shown). Thus, we examined the change in spontaneous activity of the CGCs when we perfused the CNS with 1 mmol $l^{-1}$ GABA solution. Illustrative data are shown in Fig. 3C, while data from multiple recordings of CGCs are shown in Fig. 3D. A one-way ANOVA ($F_{2,9}$=11.47; $P$=0.0033) showed that there were significant differences in the GABA effect in good learners and poor learners compared with that in naive snails. GABA had little effect on the CGCs from naive snails, while GABA effectively caused a silencing of CGC activity in good learners ($P$=0.0027). GABA also reduced CGC activity in poor learners ($P$=0.0422); however, the effect was less than that in good learners. For the mechanism of

Journal of Experimental Biology (2021) 224, jeb238055. doi:10.1242/jeb.238055

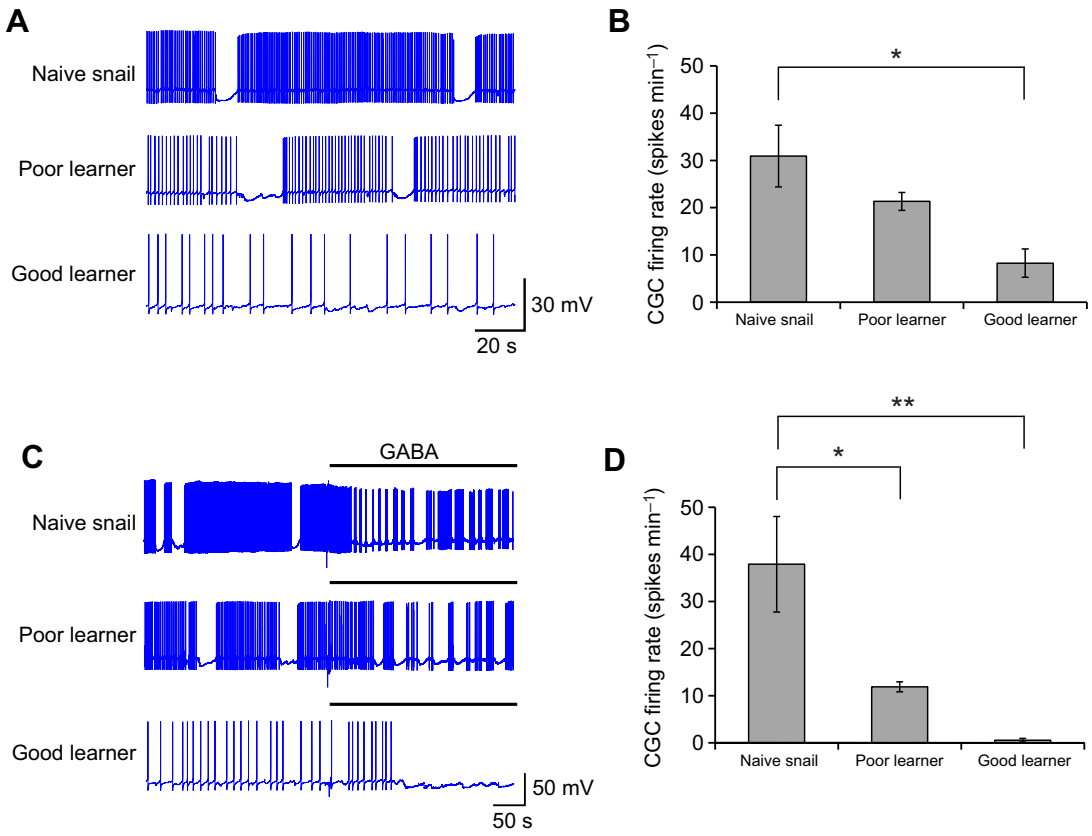


**Fig. 3. Spontaneous activity of CGCs 24 h after CTA training (without Epi).** (A) Illustrative data from the CGC of a naive snail, a poor learner and a good learner. (B) Firing rates of CGCs from naive snails ($n$=4), poor learners ($n$=3) and good learners ($n$=5). *$P$<0.05. (C) Illustrative data from CGCs following CNS perfusion with 1 mmol $l^{-1}$ GABA solution, as indicated by the horizontal bars. (D) CGC firing rate 10–13 min after the onset of GABA application in naive snails ($n$=4), poor learners ($n$=3) and good learners ($n$=5). *$P$<0.05, **$P$<0.01.

LTM formation, a series of reports previously showed that CGCs exert a weak excitatory monosynaptic and strong inhibitory post-synaptic influence upon the N1M cell, and the inhibitory post-synaptic potential (IPSP) evoked in the N1M cell is enhanced in preparations demonstrating LTM following CTA training (Kojima et al., 1997, 2001; Ito et al., 2012). However, our data are consistent with the hypothesis that a GABAergic neuron may play a significant role in mediating CTA-LTM. Further, the data are also consistent with the suggestion that the effect of Epi on the CGC may involve a GABAergic pathway. For example, the GABA sensitivity of a neuron (maybe the CGC itself) might be enhanced in good learners or in snails exposed to Epi.

Recently, CTA-LTM has been shown to involve alterations in both the monoamine and insulin content of the snails. Those previous findings can be summarized as follows. Snails with the best memory (i.e. the good learners) have the lowest monoamine content and optimal levels of molluscan insulin-related peptides (Murakami et al., 2013; Kojima et al., 2015; Aonuma et al., 2018; Totani et al., 2019, 2020). The optimal insulin levels also result in a decrease in 5-HT content (Aonuma et al., 2018). Thus, insulin-like molecules in *Lymnaea* are a necessary component for LTM formation following CTA training. To those findings, we now add that an increase in the persistence of CTA-LTM results from the effect of Epi on CGC activity and it could involve GABAergic neurons. It has also been shown in mammals, but not yet in *Lymnaea*, that compounds high in Epi, such as green tea, have a direct effect on insulin activity (Cremonini et al., 2020; Xu et al., 2020). In addition, it must be remembered that the ability to form and recall a LTM following CTA training is dependent on CGCs (Sunada et al., 2017).

**Acknowledgements**
We thank Kouya Kato and Wataru Saito, graduates of Nihon University, for their help in performing experiments.

**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
Conceptualization: M.S.; Methodology: K.L., M.S.; Formal analysis: Y.K.; Investigation: A.I., Y.K.; Data curation: A.I., Y.K.; Writing - original draft: M.S.; Writing - review & editing: K.L.; Visualization: A.I., Y.K.; Supervision: M.S.; Project administration: M.S.; Funding acquisition: M.S.

**Funding**
This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

**References**

**Abd El Mohsen, M. M., Kuhnle, G., Rechner, A. R., Schroeter, H., Rose, S., Jenner, P. and Rice-Evans, C. A.** (2002). Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radic. Biol. Med.* **33**, 1693-1702. doi:10.1016/S0891-5849(02)01137-1

**Abel, T. and Kandel, E. R.** (1998). Positive and negative regulatory mechanisms that mediate long-term memory storage. *Brain Res. Rev.* **26**, 360-378. doi:10.1016/S0165-0173(97)00050-7

**Aonuma, H., Totani, Y., Kaneda, M., Nakamura, R., Watanabe, T., Hatakeyama, D., Dyakonova, V. E., Lukowiak, K. and Ito, E.** (2018). Effects of 5-HT and insulin on learning and memory formation in food-deprived snails. *Neuro. Learn. Mem.* **148**, 20-29. doi:10.1016/j.nlm.2017.12.010

**Bailey, C. H. and Kandel, E. R.** (1993). Structural changes accompanying memory storage. *Annu. Rev. Physiol.* **55**, 397-426. doi:10.1146/annurev.ph.55.030193.002145

**Benjamin, P. R.** (2012). Distributed network organization underlying feeding behavior in the mollusk *Lymnaea*. *Neural Systems & Circuits* **2**, 4. doi:10.1186/2042-1001-2-4

**Cremonini, E., Iglesias, D. E., Kang, J., Lombardo, G. E., Mostofinejad, Z., Wang, Z. and Oteiza, P. I.** (2020). (−)-Epicatechin and the comorbidities of obesity. *Arch. Biochem. Biophys.* **690**, 108505. doi:10.1016/j.abb.2020.108505

**Fruson, L., Dalesman, S. and Lukowiak, K.** (2012). A flavonol present in cocoa [(-)epicatechin] enhances snail memory. *J. Exp. Biol.* **215**, 3566-3576. doi:10.1242/jeb.070300

**Gelperin, A., Kleinfeld, D., Denk, W. and Cooke, I. R. C.** (1996). Oscillations and gaseous oxides in invertebrate olfaction. *J. Neurobiol.* **30**, 110-122. doi:10.1002/(SICI)1097-4695(199605)30:1<110::AID-NEU10>3.0.CO;2-Q

**Ito, I., Kimura, T., Suzuki, H., Sekiguchi, T. and Ito, E.** (1999). Effects of electrical stimulation of the tentacular digits of a slug upon the frequency of electrical oscillations in the procerebral lobe. *Brain Res.* **815**, 121-125. doi:10.1016/S0006-8993(98)01115-9

**Ito, E., Otsuka, E., Hama, N., Aonuma, H., Okada, R., Hatakeyama, D., Fujito, Y. and Kobayashi, S.** (2012). Memory trace in feeding neural circuitry underlying conditioned taste aversion in *Lymnaea*. *PLoS ONE* **7**, e43151. doi:10.1371/journal.pone.0043151

**Ito, E., Yamagishi, M., Takigami, S., Sakakibara, M., Fugito, Y. and Lukowiak, K.** (2015). The Yerkes-Dodson law and appropriate stimuli for conditioned taste aversion in *Lymnaea*. *J. Exp. Biol.* **218**, 336-339. doi:10.1242/jeb.113266

**Kemenes, I., Straub, V. A., Nikitin, E. S., Staras, K., O'Shea, M., Kemenes, G. and Benjamin, P. R.** (2006). Role of delayed nonsynaptic neuronal plasticity in long-term associative memory. *Curr. Biol.* **16**, 1269-1279. doi:10.1016/j.cub.2006.05.049

**Kimura, T., Iwama, A. and Sekiguchi, T.** (1999). Contributions of superior and inferior tentacles to learned food-avoidance behavior in *Limax marginatus*. *Zool. Sci.* **16**, 595-602. doi:10.2108/zsj.16.595

**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. doi:10.1242/jeb.110726

**Kojima, S., Yamanaka, M., Fujito, Y. and Ito, E.** (1996). Differential neuroethological effects of aversive and appetitive reinforcing stimuli on associative learning in *Lymnaea stagnalis*. *Zool. Sci.* **13**, 803-812. doi:10.2108/zsj.13.803

**Kojima, S., Nanakamura, H., Nagayama, S., Fujito, Y. and Ito, E.** (1997). Enhancement of an inhibitory input to the feeding central pattern generator in *Lymnaea stagnalis* during conditioned taste-aversion learning. *Neurosci. Lett.* **230**, 179-182. doi:10.1016/S0304-3940(97)00507-7

**Kojima, S., Hosono, T., Fujito, Y. and Ito, E.** (2001). Optical detection of neuromodulatory effects of conditioned taste aversion in the pond snail *Lymnaea stagnalis*. *J. Neurobiol.* **49**, 118-128. doi:10.1002/neu.1069

**Kojima, S., Sunada, H., Mita, K., Sakakibara, M., Lukowiak, K. and Ito, E.** (2015). Function of insulin in snail brain in associative learning. *J. Comp. Physiol. A* **201**, 969-981. doi:10.1007/s00359-015-1032-5

**Li, Q., Zhao, H. F., Zhang, Z. F., Liu, Z. F., Pei, X. R., Wang, J. B., Cai, M. Y. and Li, Y.** (2009). Long-term administration of green tea catechins prevents age-related spatial learning and memory decline in C57BL/6 J mice by regulating hippocampal cyclic amp-response element binding protein signaling cascade. *Neuroscience* **159**, 1208-1215. doi:10.1016/j.neuroscience.2009.02.008

**Lukowiak, K., Ringseis, E., Spencer, G., Wilderring, W. and Syed, N.** (1996). Operant conditioning of aerial respiratory behaviour in *Lymnaea 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 pattern in *Lymnaea stagnalis*. *J. Exp. Biol.* **201**, 877-882.

**Lukowiak, K., Sangha, S., McComb, C., Varshney, N., Rosenegger, D., Sadamoto, H. and Scheibenstock, A.** (2003). Associative learning and memory in *Lymnaea stagnalis*: how well do they remember? *J. Exp. Biol.* **206**, 2097-2103. doi:10.1242/jeb.00374

**Matzel, L. D., Talk, A. C., Muzzio, I. A. and Rogerts, R. F.** (1998). Ubiquitous molecular substrates for associative learning and activity-dependent neuronal facilitation. *Rev. Neurosci.* **9**, 129-167. doi:10.1515/REVNEURO.1998.9.3.129

**Mocca, F., Di Cristo, C., Winlow, W. and Di Cosmo, A.** (2009). $GABA_A$- and AMPA-like receptors modulate the activity of an identified neuron within the central pattern generator of the pond snail *Lymnaea stagnalis*. *Invertebr. Neurosci.* **9**, 29-41. doi:10.1007/s10158-009-0086-x

**Murakami, J., Okada, R., Sadamoto, H., Kobayashi, S., Mita, K., Sakamoto, Y., Yamagishi, Y., Hatakeyama, D., Otsuka, E., Okuta, A. et al.** (2013). 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. doi:10.1523/JNEUROSCI.0679-12.2013

**Romanava, E. V., Rubakhin, S. S. S.-rózsa, K.** (1996). Behavioral changes induced by GABA-receptor agonists in *Lymnaea stagnalis* L. *Gen. Pharmacol.* **27**, 1067-1071. doi:10.1016/0306-3623(95)00122-0

**Scheibenstock, A., Krygier, D., Haque, Z., Syed, S. and Lukowiak, K.** (2002). The soma of RPeD1 must be present for long-term memory formation of associative learning in *Lymnaea*. *J. Neurophysiol.* **88**, 1584-1591. doi:10.1152/jn.2002.88.4.1584

**Spenser, G. E., Syed, N. I. and Lukowiak, K.** (1999). Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnaea stagnalis*. *J. Neurosci.* **19**, 1836-1843. doi:10.1523/JNEUROSCI.19-05-01836.1999

**Sugai, R., Azami, S., Shiga, H., Watanabe, T., Sadamoto, H., Kobayashi, S., Hatakeyama, D., Fujito, Y., Lukowiak, K. and Ito, E.** (2007). One-trial conditioned taste aversion in *Lymnaea*: good and poor performers in long-term memory acquisition. *J. Exp. Biol.* **210**, 1225-1237. doi:10.1242/jeb.02735

**Sunada, H., Lukowiak, K. and Ito, E.** (2017). Cerebral giant cells are necessary for the formation and recall of memory of conditioned taste aversion in *Lymnaea*. *Zool. Sci.* **34**, 72-80. doi:10.2108/zs160152

**Swinton, E., de Freitas, E., Swinton, C., Shymansky, T., Hiles, E., Zhang, J., Rothwell, C. and Lukowiak, K.** (2018). Green tea and cocoa enhance cognition in *Lymnaea*. *Commun. Integr. Biol.* **11**, e1434390. doi:10.1080/19420889.2018.1434390

**Swinton, C., Swinton, E., Shymansky, T., Hughes, E., Zhang, J., Rothwell, C., Kakadiya, M. and Lukowiak, K.** (2019). Configural learning: a higher form of learning in *Lymnaea*. *J. Exp. Biol.* **222**, jeb190405. doi:10.1242/jeb.190405

**Totani, Y., Aonuma, H., Oike, A., Watanabe, T., Hatakeyama, D., Sakakibara, M., Lukowiak, K. and Ito, E.** (2019). Monoamines, insulin and the roles they play in associative learning in pond snails. *Front. Behav. Neurosci.* **13**, 65. doi:10.3389/fnbeh.2019.00065

**Totani, Y., Nakai, J., Dyakonova, V. E., Lukowiak, K., Sakakibara, M. and Ito, E.** (2020). Induction of LTM following an insulin injection. *eNeuro* **7**, ENEURO.0088-20.2020. doi:10.1523/ENEURO.0088-20.2020

**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. doi:10.1523/JNEUROSCI.0914-07.2007

**Wertz, P. W.** (2013). Current understanding of skin biology pertinent to skin penetration: Skin biochemistry. *Skin Pharmacol. Physiol.* **26**, 217-226. doi:10.1159/000351949

**Yamanaka, M., Sadamoto, H., Hatakeyama, D., Nakamura, H., Kojima, S., Kimura, T., Yamashita, M., Urano, A. and Ito, E.** (1999). Developmental changes in conditioned taste aversion in *Lymnaea stagnalis*. *Zool. Sci.* **16**, 9-16. doi:10.2108/zsj.16.9

**Yeoman, M. S., Pieneman, A. W., Ferguson, G. P., Ter Maat, A. and Benjamin, P. R.** (1994a). Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail, *Lymnaea*. I. Fine wire recording in the intact animal and pharmacology. *J. Neurophysiol.* **72**, 1357-1371. doi:10.1152/jn.1994.72.3.1357

**Yeoman, M. S., Kemenes, G., Benjamin, P. R. and Elliott, C. J.** (1994b). Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail, *Lymnaea*. II. Photoinactivation. *J. Neurophysiol.* **72**, 1372-1382. doi:10.1152/jn.1994.72.3.1372

**Yeoman, M. S., Brierley, M. J. and Benjamin, P. R.** (1996). Central pattern generator interneurons are targets for the modulatory serotonergic cerebral giant cells in the feeding system of *Lymnaea*. *J. Neurophysiol.* **75**, 11-25. doi:10.1152/jn.1996.75.1.11

**Xu, R., Bai, Y., Yang, K. and Chen, G.** (2020). Effects of green tea consumption on glycemic control: a systematic review and meta-analysis of randomized controlled trials. *Nutr. Metabol.* **17**, 56. doi:10.1186/s12986-020-00469-5