Chapter

Green Tea-Derived Catechins Have Beneficial Effects on Cognition in the Pond Snail

Yoshimasa Komatsuzaki, Ayaka Itoh and Minoru Saito

Abstract

Green tea has been used as a medicine in East Asia for thousands of years. Plant-derived compounds called flavanols, which are included in green tea, may have potentials to help maintain healthy brain function. In this chapter, we review the effects of flavanols, e.g. epicatechin (EpiC), on cognitive ability in the pond snail, *Lymnaea stagnalis*. In this decade, the Lukowiak's group has tested the effects of EpiC on cognition ability in *Lymnaea*. In a *Lymnaea* model system, they showed that EpiC and EpiC-containing foods have a rapid and activity-dependent effect enhancing the formation of long-term memory (LTM) following operant conditioning of aerial respiratory behavior. In the last part of this chapter, we also introduce our study for the effects of EpiC on LTM formation in another model system in *Lymnaea*. This study showed that EpiC increases the persistence of LTM formed by classical conditioning of feeding behavior, and suggested that EpiC alters some electrophysiological properties of a neuron in the feeding system.

Keywords: Green tea-derived catechins, Epicatechin, Operant conditioning, Classical conditioning, Learning and memory, Long-term memory, *Lymnaea*

1. Introduction

Green tea is one of the most popular beverages in the world. It is made from *C. sinensis* leaves and include many kinds of phytochemicals. Compounds called flavanols, which are included in green tea, are candidates for the active ingredient that has been used as a medicine in East Asia for thousands of years. An extract, called Sinecatechins, of green tea leaves is also used as botanical drug approved by the FDA in USA [1]. Flavanols (catechins) belonging to the group of polyphenols [2] are contained in green tea (mg/100 g): 26.05 (−)-epigallocatechin-3-gallate (EGCG), 7.57 (−)-epicatechin-3-gallate (ECG), 16.02 (−)-epigallocatechin (EGC) and 6.16 (−)-epicatechin (EpiC) [3]. Recently, it is reported that flavanols may have potentials to help maintain healthy brain function in both vertebrates and invertebrates [4–8].

Gastropod mollusks such as *Aplysia*, *Limax*, *Hermissenda* [9–14] and *Lymnaea* [15–19] are excellent model animals for understanding the causal neuronal mechanisms of learning and memory. In this decade, the Lukowiak's group in University of Calgary has tested the effects of EpiC on cognition in *Lymnaea stagnalis.* In a *Lymnaea* model system, they showed that EpiC and EpiC-containing foods have a

rapid and activity-dependent effect enhancing the formation of long-term memory following operant conditioning of aerial respiratory behavior.

Lymnaea is an aquatic pulmonate snail and can breathe either with its lung or skin. It approaches water surface and gets air into the lung through opening its pneumostome (**Figure 1A**). The Lukowiak's group employed a protocol of operant conditioning of aerial respiration to investigate the cognitive function in *Lymnaea*. In a hypoxic environment in which the frequency of aerial respiration in *Lymnaea* increases, applying repeated tactile stimulus to pneumostome as a negative reinforcement (training session; TS) reduces the number of attempted pneumostome openings in *Lymnaea*, and then the behavioral change persists for 3 hours or longer.

A single 30-min training session (0.5 h-TS) results in intermediate-term memory (ITM) that persists for up to 3 hours, whereas two 30-min training sessions with a 1 hour rest interval (2 h-TS) results in long-term memory (LTM) that persists for 24 hours [21]. ITM depends on the translation of existing mRNA transcripts but does not require mRNA transcription. LTM requires both the translation of mRNA and the formation of new mRNA transcripts [22]. Thus, a 2 h-TS could drive the process of mRNA transcription in addition to translation of mRNA.

To drive aerial respiration in *Lymnaea*, a 3-neuron central pattern generator (CPG) was shown to be both necessary and sufficient (**Figure 1B**) [23, 24]. Subsequently, it was shown that one of the three CPG neurons, Right Pedal Dorsal 1 (RPeD1), is a necessary site for LTM formation, extinction and reconsolidation of the memory [18, 22, 25]. It is also possible to utilize a semi-intact preparation where aerial respiratory behavior and neuronal activity can be simultaneously studied [26, 27].

Lymnaea can be classically, as well as operantly, conditioned and LTM can be formed by the following learning procedures [28, 29]. Conditioned taste aversion (CTA), which is a classical conditioning, is based on pairing sucrose as a conditioned stimulus (CS) with an aversive chemical unconditioned stimulus (UCS) such as KCl, which inhibits feeding and evokes a withdrawal response. After ten trials, the feeding response of trained snails to sucrose became significantly weaker than that of control snails, and this associative memory lasted for more than 2 weeks [30].

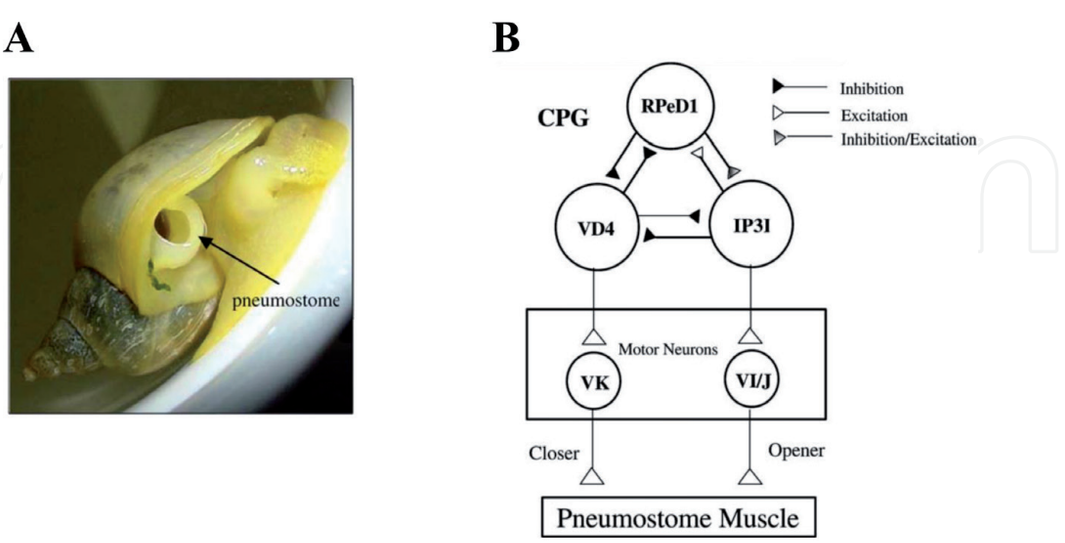

Figure 1.
Pneumostome in Lymnaea *and the neural circuit including in aerial respiratory behavior. (A)* Lymnaea *with the opened pneumostome. (B) Schematic drawing of the central pattern generator (CPG) to drive aerial respiration. Depolarization of Right Pedal Dorsal 1 (RPeD1) activates input3-interneuron (IP3I) via a biphasic effect (inhibition followed by excitation). Subsequently, IP3I excites both RPeD1 and a group of motor neurons (VI/J cells) involved in pneumostome opening. IP3I also inhibits visceral dorsal 4 interneuron (VD4), which is involved in pneumostome closing. The combined inhibitory input from both RPeD1 and IP3I causes burst firing of VD4. These figures are reproduced from [20] with permission.*

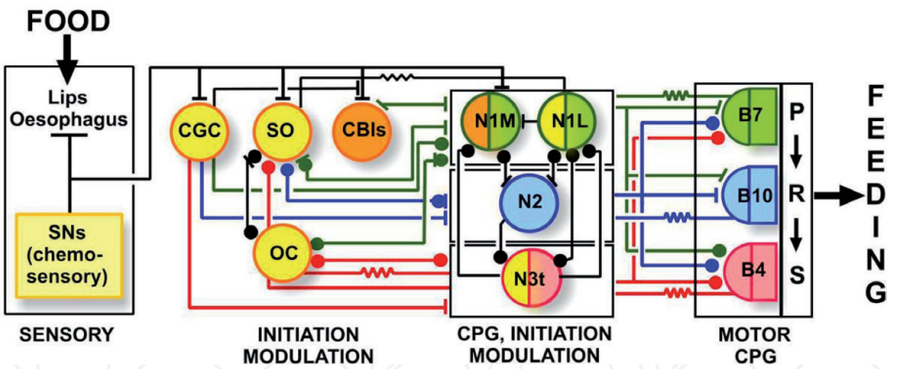

Figure 2.
Neuronal circuit corresponding to the feeding behavior. Each neuron is indicated by an abbreviation (see [31]). Modulatory function is indicated by yellow and initiating function by orange. Central pattern generator (CPG) interneurons and motoneurons active during the three phases of the feeding rhythm are indicated by green (P = protraction), blue (R = rasp) and red (S = swallow). Neurons labeled with two colors have two functions. Dots indicate inhibitory chemical synapses, bars excitatory chemical synapses and resistor symbols electrotonic (electrical) synapses. This figure is reproduced from [31] with permission.

Individual neurons in *Lymnaea* can be identified as the neuronal circuit corresponding to the feeding behavior (**Figure 2**). An identified spontaneously active pair of neurons, the cerebral giant cells (CGCs), has been shown to both modulate the neuronal network underling the feeding behavior and be necessary for LTM and its retrieval following CTA training [31, 32]. The most significant CGC synaptic connections are with the neuron 1 medial (N1M) cell, an interneuron in the CPG that co-ordinates rhythmic feeding movements [33–36].

In the last part of this chapter, we introduce our study for the effects of EpiC on LTM formation in the feeding system in *Lymnaea*.

2. Enhancing effects of epicatechin on memory formation by operant conditioning of aerial respiratory behavior

To date, the most detailed information on effects of EpiC in *Lymnaea* has been obtained from experiments using operant conditioning of aerial respiratory behavior. In this paradigm, snails are subjected to a protocol that the pneumostome receives a weak tactile stimulus whenever the snail attempts to open the pneumostome in a hypoxic environment. The number of attempted pneumostome openings is recorded for each snail for 30 minutes. To determine whether memory is formed following the training session (TS), an identical procedure is performed 24 hours later, which is called a memory test (MT). The number of attempted pneumostome openings in the MT is compared with that in the TS, and long-term memory (LTM) is evaluated if the number of attempted openings in the MT is significantly lower than that in the TS [25, 37].

When snails were given a 0.5 h-TS, which do not usually form LTM lasting 24 hours or more, in the presence of 15 mg/L EpiC, the memory persisted until 24 hours after that training. Thus, EpiC can promote LTM formation by driving the process of mRNA transcription in addition to mRNA translation. EpiC also enhances LTM formation. When snails were operantly conditioned in EpiC-containing pond water (15 mg/L) by a 2 h-TS (TS1, TS2; **Figure 3A**), which typically results in memory lasting only 24 hours in pond water without EpiC (control group, TS1 versus MT, n = 12, no significance; **Figure 3B**), they formed LTM lasting at least 72 hours (EpiC group, TS1 versus MT, n = 12, $P < 0.01$; **Figure 3C**) [4, 38]. Moreover, following a 2 h-TS in EpiC-containing pond water, snails were received the MT in standard pond water (i.e. no EpiC) at 96 hours, 1 week and 2 weeks

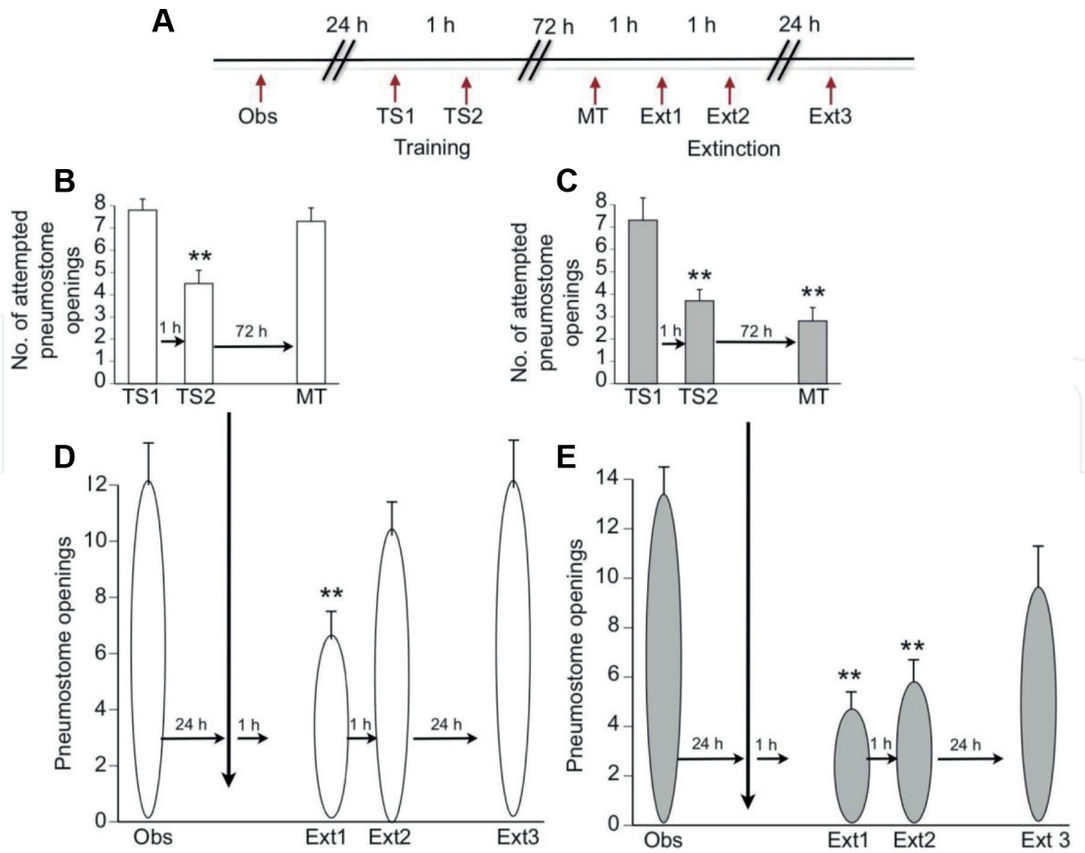

Figure 3.
*Epicatechin (EpiC) enhances LTM formation and diminish the rate of extinction following operant conditioning. (A) A timeline of the experiment is shown. (B–E) White and gray bars show snails trained without EpiC or with EpiC, respectively. Snails were either operantly conditioned in pond water (B) or EpiC-containing pond water (C). (D, E) In an observation (Obs) and extinction sessions (Ext1–3), the number of pneumostome openings was calculated for each snail without tactile stimulation in hypoxic water for 30 minutes. (B, C) n = 12, **P < 0.01 compared with TS1. (D, E) n = 12, **P < 0.01 compared with Obs. These figures are produced from [4] with permission.*

after the 2 h-TS [38]. The snails maintained in a very long-term memory lasting for 2 weeks or longer. Memory formed in the presence of EpiC is resistant to forgetting, which is not dependent on EpiC being present in the MT. In addition, snails received extinction sessions (Ext1–3). In extinction sessions, snails are allowed to freely perform aerial respiration in the same hypoxic environment as TS, and the learned association between the pneumostome opening and weak tactile stimulus can be extinguished. Snails trained in the presence of EpiC were also more resistant to extinction of memory (EpiC group, Obs (pre-training) versus Ext2, n = 12, $P < 0.01$; **Figure 3E**), including blocking "the original memory" by overwriting a newer memory [39, 40], than control snails (control group, Obs (pre-training) versus Ext2, n = 12, no significance; **Figure 3D**) [4].

Exposure to EpiC does not alter locomotor activity and spontaneous aerial respiratory behavior themselves in *Lymnaea* compared to naïve snails [4]. Thus, studying the effects of EpiC could exemplify specificities for drugs that directly interact with neuronal signaling pathways for LTM formation and persistent.

In mice, EpiC improves retention of spatial memory by enhancing angiogenesis [8]. Oral intake of EpiC via gavage increases the level of EpiC and its metabolites in rat plasma and brain [41], and then EpiC could influence brain functions. In *Lymnaea*, EpiC can easily absorb via skin into the body cavity, and subsequently may contact to the CNS by an open circulatory system.

Additionally, it is reported that EpiC also has a rapid and activity-depend effect on promoting LTM formation in *Lymnaea* [4, 5, 38]. Fernell et al. demonstrated that

EpiC must be present during operant conditioning or applied with 1 hour immediately after training in order for EpiC to cause LTM enhancement [42]. However, EpiC exposure at 1 hour after training did not result in enhanced memory formation. Thus, One hour after training is important for LTM formation. It is widely known that there is an important period for encoding and consolidation of the memory following learning [43]. In *Lymnaea*, the consolidation period persists for about 1 hour. LTM requires both regulation of gene activity and new protein synthesis, whereas ITM requires only new protein synthesis. Previously, it was shown in *Lymnaea* that applying a cold block (10 minutes in 4°C pond water) immediately after training blocks only LTM formation, but not block ITM formation [44, 45]. Thus, these results suggest that EpiC could alter the gene activity for LTM.

A specific mechanism by which EpiC enhances memory formation has not been clarified. However, there have been many studies in mammals suggesting various ways in which dietary flavonoids may exert such beneficial effects on the CNS [8]. EpiC is known as antioxidant, protecting neurons from injury caused by oxidative stress [46]. EpiC can be photo-inactivated by exposure to ultraviolet light (UV). Exposed to the sun for 6 hours, EpiC changes the molecular conformation by breaking the cyclic ether through a radical mechanism [47]. But no significant change was observed in the antioxidant activities of EpiC upon 6 h beta-UV radiation. Following photo-inactivation of EpiC, memory enhancement did not occur. Photo-inactivation of foods containing EpiC also blocked their ability to enhance LTM [7]. Thus, enhancing effect of EpiC on memory formation in *Lymnaea* is less likely to be caused by antioxidant properties of EpiC and may be directly due to affecting the signaling pathway required for memory formation in neurons. This does not mean that antioxidant properties do not act against oxidative damage and therefore protective effect may contribute to retain memory over long-term.

As mentioned above, LTM formation is dependent on altered gene activity and new protein synthesis [48]. It is well-known that the consolidation period following learning plays an important role for LTM formation [20, 49], in which the learning is encoded into memory. EpiC can cause an enhancement of memory formation if snails experience EpiC during training or immediately after training. However, EpiC exposure at 1 hour before training or 1 hour after training was not sufficient to cause memory enhancement [42]. In *Lymnaea*, it is thought that the consolidation period persists for about 1 hour. If the operant conditioning is performed in the presence of environment stressors (exposure to predator kairomones or KCl), it results in strengthening of memory formation. The effects via sensory input from the osphradium (a sensory organ) are dependent on a serotonergic signaling pathway [50]. However, the enhancing effects of EpiC do not require either the input from the osphradium or serotonergic signaling pathway.

3. Signaling pathway involved in epicatechin effect

EpiC effects on LTM formation may be due to its ability to drive an increase in intracellular kinase activity [51] in neurons such as RPeD1. It has also been shown that activation of CREB is necessary for LTM formation in *Lymnaea* [52] and EpiC increases CREB-regulated gene expression in neurons [53]. Further, there is increasing evidence implying that EpiC can drive rapid signaling intracellular as it increases phosphorylation of protein kinase B (Akt)/PI3K, PKC and Erk MAPK and induces cellular survival/proliferation in human hepatoma cells [54]. This is important since LTM following operant conditioning in *Lymnaea* requires activations of PKC and MAPK [55]. In addition, EpiC appears to be able to directly alter DNA methylation activity [56], which has been shown in *Lymnaea* to alter LTM formation [57]. EpiC

has been shown in the mammalian brain to cross the blood brain barrier and directly affect CNS function possibly by enhancing 5HT function [58]. EpiC may also activate NOS and stimulate NO production [53]. It is known in the *Lymnaea* model system that 5HT and NO are involved in LTM formation [50, 59]. It remains to be elucidated whether EpiC brings about its enhancing effects on LTM formation via these molecules. EpiC effects on cognitive enhancement in mammalian preparations has been shown, but it is unclear whether the enhanced cognitive benefit is directly due to altering neuronal activity or through effects on blood flow to the brain as a result of increased angiogenesis [8].

It is reported that exposure to crayfish effluent (CE), which also enhances LTM formation and significantly decreases RPeD1 excitability [60], works a serotonergic pathway that can be blocked by mianserin, a serotonin receptor antagonist [50]. As previously shown, however, mianserin does not affect the LTM formation enhancement induced by EpiC [4]. In addition, once the osphradial nerve that connects the osphradium (a sensory organ) to the CNS is severed, CE no longer enhances LTM formation [50]. Thus, the osphradial nerve must be intact in order to cause enhancement of LTM formation by exposing CE. Whereas, EpiC enhanced LTM formation after severing the osphradial nerve [4]. Thus, it appears that EpiC acts via a different mechanism and a different pathway from those caused by the perception of CE.

McComb and collaborators demonstrated the memory formation by using *in vitro* semi-intact preparations [26]. After operant conditioning of intact snails, semi-intact preparations were dissected so that changes in the respiratory behavior (pneumostome openings) and underlying activity of the identified CPG neuron, RPeD1, could be monitored simultaneously.

Our group can perform "*in vitro*" operant conditioning in semi-intact preparations from naïve snails. In the training, we applied a gentle tactile stimulus to the pneumostome area whenever the snail began to open it. Following the training, the respiratory behavior decreased. After the training, naïve snails exposed to EpiC (15 mg/L) prior to recording exhibited significantly increased RPeD1 excitability compared with non-exposed snails. This experiment can help to understand how EpiC alters RPeD1 excitability to drive aerial respiratory behavior and leads to enhanced LTM formation.

These results provide the basis of future studies in *Lymnaea* to elucidate how EpiC enhances LTM formation of respiratory conditioning.

4. Effects of intaking of catechin-rich foods

Does exposure to food products containing EpiC during the training elicit similar effects seen for exposure to pure EpiC? Lukowiak et al. demonstrated interesting experiments whether foods containing substantial amounts of EpiC, such as green tea, cocoa, apple peel and black tea, can enhance memory formation in *Lymnaea* [7]. Exposed to pond water containing green tea or pure cocoa powder in concentration comparable to human consumption level (approximately 1 g/day) during training, the memory enhancement was comparable to that elicited by pure EpiC experiments [7].

Interestingly, black tea does not only enhance LTM formation but suppresses LTM formation in *Lymnaea* [61]. Black tea is made from the same plant as green-tea through an oxidation process called "fermentation" and becomes stronger in flavor than green tea. However, the content of EpiC in black tea (0.49 mg/100 g) reduces compared with green tea (6.16 mg/100 g) [3]. Black tea substantially contains more other flava-3-nols, thearubigins and theaflavins, than green tea [3, 62]. These flava-3-nols are formed during the fermentation reaction in black tea. As far as we

know, no studies have investigated direct effects of these flava-3-nols on memory formation. However, it is reported that intake of theaflavins is associated with long-term language and verbal memory in human [63]. In another study, theaflavins are reported to improve memory impairment [64, 65]. Thus, thearubigins and theaflavins may be not candidate substances for blocking memory formation in black tea.

Another component of black tea, caffeine, can inhibit cognitive function. In drosophila, caffeine reduces the performance for light aversive conditioning [66]. However, both green tea and black tea contain a same amount of caffeine. It is possible that L-theanine included in green tea in comparatively large amounts is thought to balance the effects of caffeine. The combination of these two substances may be synergistic, as one study found that people who ingested L-theanine and caffeine together had better attention than when either was used alone [67, 68]. Therefore, investigating catechin-rich foods is difficult to permit a full understanding of the specific effect of these phytochemicals.

5. Rescue effect of epicatechin on stress-impaired memory

Green tea-derived catechins do not only enhance memory formation, but also rescue impaired cognitive functions due to environmental stressors. Catechin-rich foods have been considered to improve various aspects of cognitive functions in rodents and humans, and some reports suggest that it has positive effects on mild cognitive impairment [69–71]. EpiC administration improves spatial memory in mice via an increase in cerebral angiogenesis or a direct effect on neural elements [8]. In *Lymnaea*, there are some reports for the recovery effect of EpiC on impaired function by environmental stressor [5, 38].

Most of freshwater mollusks including *Lymnaea* are dependent on calcium intake directly from the environment through their skin [72] and exhibit reduced shell growth in the environment containing less than 20 mg/L calcium [73, 74]. It is considered that this level of calcium acts as a stressor on the snail. Following 1 h exposure to a low calcium environment, *Lymnaea* was not able to form LTM, although it still had an ability of ITM [75]. Following a 2 h-TS in EpiC-supplemented low-calcium pond water, snails persist a decrease of respiratory behavior both 24 hours and 72 hours after the training [5]. In addition, memory formation of the training in EpiC-suppelmented pondwater was not diminished by the combination of a low- calcium pond water environment and 1 hour of crowding immediately prior to operant conditioning training, which blocks all forms of memory (short-term, intermediate-term and long-term memory) in *Lymnaea* [38]. These results suggest that EpiC reverses an imposed memory deficit by exposure to memory 'unfriendly' stress.

6. Enhancing effect of epicatechin on memory formation by classical conditioning of feeding behavior

Lymnaea can be classically, as well as operantly, conditioned [28, 29]. Conditioned taste aversion (CTA) is a classical conditioning, which is based on pairing sucrose as a conditioned stimulus (CS) with an aversive chemical unconditioned stimulus (UCS) such as KCl, which inhibits feeding and evokes a withdrawal response. After this procedure, trained snails show a significantly weaker feeding response to sucrose than controls. We here introduced the enhancing effect of EpiC on LTM formed by CTA. In the previous study, we showed that EpiC increases the persistence of LTM as mentioned below [76].

CTA training procedure we performed is briefly as follows. Adult snails randomly chosen were food deprived for 24 hours before being subjected to CTA training. Snails were then immersed in an appetitive solution (10 mM sucrose) for 15 s. Then, the sucrose solution was quickly replaced with distilled water, and the feeding response (i.e. number of bites) was measured in distilled water for 5 minutes (pre-test). Ten minutes after the pre-test, CTA training was performed. In CTA training, snails were immersed for 15 s in 10 mM sucrose, which were immediately immersed for 15 s in 10 mM KCl solution (i.e. the UCS). The UCS

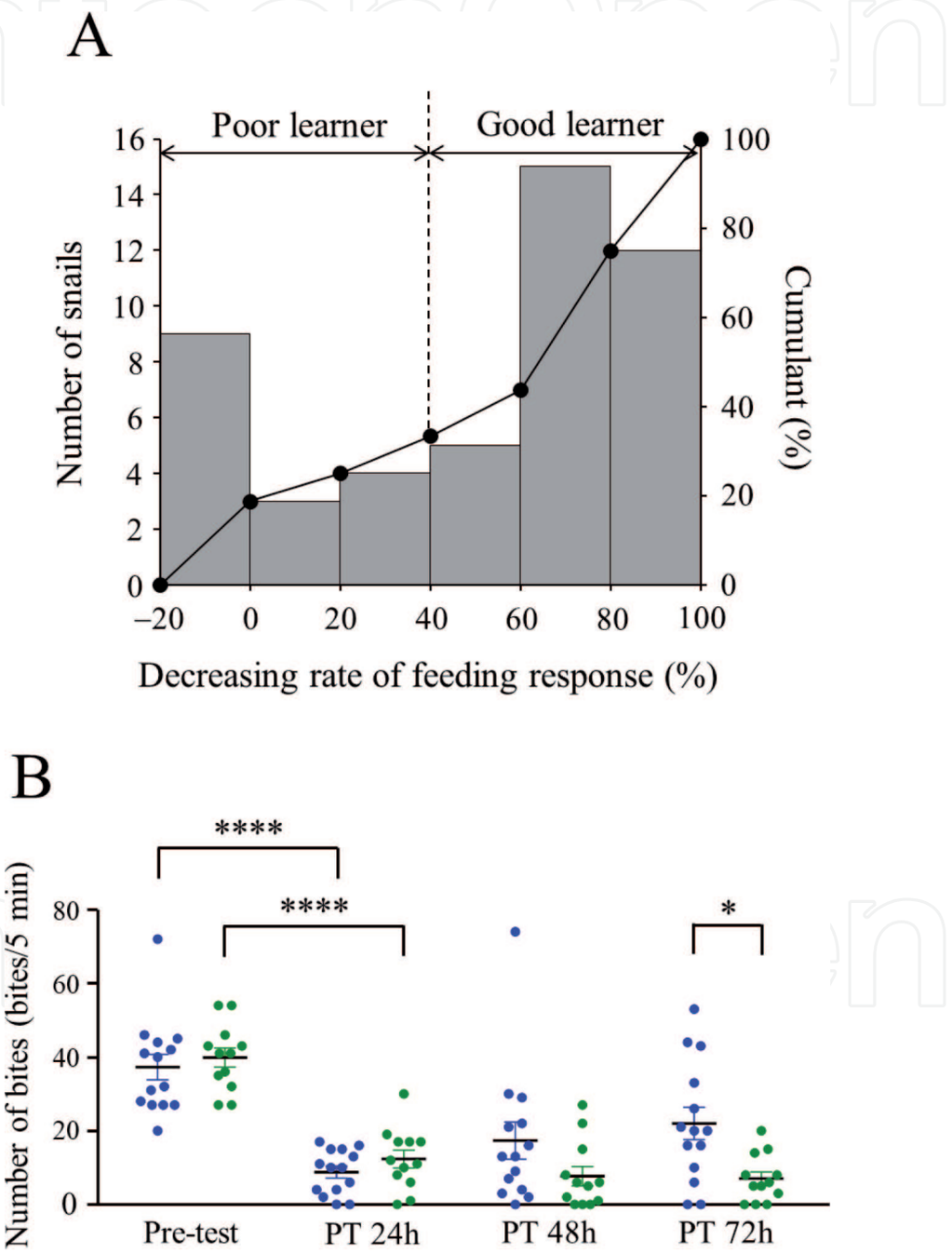

Figure 4.
*Change in feeding response after conditioned taste aversion (CTA) training. (A) Histogram showing the decreasing rate of the feeding response at the 24 h post-test n = 48). The cumulant (the ratio of the cumulative number of snails at each rate, from low to high, out of the total number, n = 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. These figures are reproduced from [76] with permission.*

.inhibits the feeding response. After the UCS was presented, snails were immersed either in distilled water (control) or EpiC solution (15 mg/L) for 9.5 minutes. This procedure was repeated 5 times. After CTA training, snails were kept in distilled water for 24, 48 or 72 hours and then the post-test was performed, which was exactly the same as the pre-test. 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.

Figure 4A shows a histogram of the decreasing rate of the feeding response in the 24 h post-test (i.e. 24 hours after training). The decreasing rate was measured for 26 snails trained without EpiC and 22 snails trained with EpiC, and the data from all snails (n = 48) are combined in the histogram. As shown in **Figure 4A**, from their responses, the snails were roughly divided into two groups: 'good' and 'poor' learners. Good learners were defined as snails that showed at least a 40% decrease in the number of bites in the 24 h post-test compared with that in the pre-test. Thus, poor learners were defined as snails whose post-test scores decrease by less than 40%. In this data, 32 of the 48 snails (i.e. 67%) were classified as good learners.

For the good learners, we statistically analyzed on the data presented in **Figure 4B** (control group, n = 14; EpiC group, n = 12). In both groups, snails showed a significant decrease in the number of bites in the 24 h post-test (control group, 37.3 ± 3.5 to 8.8 ± 1.6 bites per 5 min, $P < 0.0001$; EpiC group: 39.9 ± 2.6 to 12.3 ± 2.4 bites per 5 min, $P < 0.0001$). Thus, placing snails in the EpiC solution in CTA training did not alter their 24 h memory performance.

We next compared the post-test scores between two groups (control group versus EpiC group) at 24, 48 and 72 hours after CTA training. A statistical analyze showed that there was a significant difference in the memory scores at 72 hours between the two groups (control group versus EpiC group, $P < 0.05$) while there was no significant difference in the 24 h and 48 h post-tests. Thus, we concluded that exposing snails to EpiC solution resulted in significantly longer memory persistence.

An identified spontaneously active pair of neurons, the cerebral giant cells (CGCs), has been shown to both modulate the neuronal network underling the feeding behavior and be necessary for LTM and its retrieval following CTA training (**Figure 4**) [31, 32]. Therefore, a possible mechanism underlying the significant effect of EpiC on LTM persistence is an alteration in CGC activity. Our data supported this possibility [76]. Additionally, our data suggested that a GABAergic neuron may play a significant role in mediating CTA-LTM and the EpiC effect on the CGC may involve a GABAergic neuron. For example, the GABA sensitivity of a neuron (maybe the CGC itself) might be enhanced in good learners or in snails exposed to EpiC.

7. Conclusion

Studies described in this chapter have provided valuable information on a possibility of EpiC-rich foods contributing to cognition ability in *Lymnaea*. In addition, animal models in *Lymnaea* contribute to new evidences for the generality of mechanisms for the effects of EpiC on learning and memory formation, across learning paradigms (e.g. classical or operant conditioning).

These studies suggest that EpiC has not only antioxidant properties but also targets molecules (e.g. specific receptors) directly to affect the signaling pathway. Then, the results may yield the basis of future studies to elucidate how EpiC enhances LTM formation of classical and operant conditioning in *Lymnaea*.

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