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Memory block: A consequence of conflict resolution

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ABSTRACT

In *Lymnaea*, experiments showed that one-day food deprivation before aversive classical conditioning results in optimal conditioned taste aversion (CTA) and long-term memory (LTM), whereas 5-day food deprivation before training did not. We hypothesized that snails do in fact learn and form LTM when trained after prolonged food deprivation, but that severe food deprivation blocks their ability to express memory. We trained 5-day food-deprived snails under various conditions, and found that memory was indeed formed but overwhelmed by severe food deprivation. Moreover, CTA-LTM was context-dependent and could be observed only when the snails were in a context similar to that in which the training occurred.

KEY WORDS: Conditioned taste aversion, Context, Food deprivation, Long-term memory, *Lymnaea*

INTRODUCTION

The pond snail *Lymnaea stagnalis* can be both classically and operantly conditioned and following the acquisition of learning forms memory (Ito et al., 2013). Snails are able to consolidate the associative learning into long-term memory (LTM) (Benjamin et al., 2000; Otsuka et al., 2013; Takahashi et al., 2013; Lukowiak et al., 2014, Sunada et al., 2014). Here we look at the ability of *Lymnaea* to learn and remember for many days not to respond to a food substance that normally elicits a feeding response. This is referred to as conditioned taste aversion (CTA) (Ito et al., 1999, 2012, 2015; Kawai et al., 2004; Sugai et al., 2006; Takigami et al., 2014). To produce a CTA, we repeatedly pair an appetitive stimulus (e.g., sucrose; the conditioned stimulus (CS)) with an aversive stimulus (e.g., electric shock; the unconditioned stimulus (US); Takigami et al., 2013; Ito et al., 2015). Application of the CS to the lips in naive snails increases the feeding response, whereas application of the US causes snails to withdraw into their shell and terminate feeding. After repeated forward temporal contingent presentations of the CS and US, the CS no longer elicits the feeding response, and this taste aversion (i.e., CTA) persists for more than a month (Kojima et al., 1996).

It appears that some degree of food-deprivation-stressed state must exist in order for the CTA to successfully occur. However, the length of food deprivation alters learning and LTM formation. For example, 1-day food deprivation resulted in the best learning and memory. Most interesting to us, however, was the finding that 5-day food deprivation before training resulted in little or no learning and memory possibly because of an overly stressed state (Sugai et al., 2007; Mita et al., 2014a,b).

We were perplexed as to why the 5-day food-deprived snails do not exhibit CTA. We hypothesized that these snails do in fact learn and form LTM but that an overly stressed state associated with prolonged food deprivation blocks their ability to express LTM when the CS is applied. In these severely food-deprived snails, there is a conflict between memory and the desire/necessity to eat. The snail has to resort to a conflict resolution process to either eat or not to eat. In a sense, the snail engages in the concept of ‘necessity knows no law’. That is, hunger triumphs over the memory not to respond

to the CS. In addition, we also hypothesized that the context-specificity of memory expression (Haney & Lukowiak, 2001) plays an important role in the lack of memory LTM expression seen in 5-day severely food-deprived snails.

RESULTS

Definition of food deprivation status

Food deprivation status was defined in the following manner:
The day when snails began food deprivation is called Day 0.
Day -1 snails: Snails were fed *ad libitum* access to food. They were not food-deprived.
Day 1 snails: Snails were food-deprived for 1 day.
Day 5 snails: Snails were food-deprived for 5 days.
After Day 5, snails had *ad libitum* access to food (turnip leaves and Spiral Shell Food).
Day 12 snails: Snails had *ad libitum* access to food for an additional 7 days.
Day 13 snails: Snails were food-deprived for an additional 1 day.
Snails were deemed to be healthy under all the above conditions as they continued to exhibit normal homeostatic behaviors such as aerial respiration, copulation and egg-laying behaviors. For example, we compared the number of eggs laid within the 24-h period following a pond water change in aquaria between Day -1 and Day 5 snails, We did not find any significant difference in these number of eggs laid (25.7 ± 2.3 eggs from 17 out of 20 Day -1 snails, and 27.5 ± 2.3 eggs from 16 out of 20 Day 5 snails; not significant by unpaired *t*-test).

The following abbreviations were used in the figures:
AF: The snails were fed *ad libitum* on turnip leaves and Spiral Shell Food.
FD: The snails were food-deprived.
TAT: The snails were taste-aversion trained.
PT: The snails were posttested.

Salience of conditioned stimulus in severely food-deprived snails

We previously demonstrated that snails that were food-deprived for 5 days (i.e., Day 5 snails) did not exhibit LTM following taste aversion training (Sugai et al., 2007; Mita et al., 2014a,b). One possibility to account for this result is that the CS (a 10 mmol l^{-1} sucrose solution) used in those studies was more salient in 5-day food-deprived snails than in control snails. That is, the same CS will evoke a greater feeding response in a 5-day food-deprived snail than in a snail that is food-deprived for a shorter period of time or not food-deprived. To determine if this is the case, we compared the number of bites per minute elicited by the application of the 10 mmol l^{-1} sucrose solution to Day -1 (snails with *ad libitum* access to food), Day 1 (snails with modest food deprivation) and Day 5 (snails with severe food deprivation) snails. The results were 12.6 ± 0.3 bites/min for Day -1 snails; 13.0 ± 0.4 bites/min for Day 1 snails; and 11.9 ± 0.2 bites/min for Day 5 snails. There were 40 snails in each of the groups. There was no significant difference in the number of bites in each group elicited by the CS ($F_{2,117}$ = 2.874, P > 0.05). That is, food deprivation had no significant effect on the feeding behavior elicited by the CS.

Aversiveness of unconditioned stimulus in severely food-deprived snails

It is possible that because the stress state of Day 5 snails is different from that of Day -1 and Day 1 snails, the perception of the US is different. For example, the aversiveness of the US could be less in Day 5 snails than in Day -1 and Day 1 snails, resulting in the US being a less potent stimulus. We therefore determined the length of time a snail stayed in its shell following the presentation of the US. A more aversive stimulus will cause snails to stay withdrawn a longer period of time. The time of emergence after US application was 27.9 ± 1.5 s for Day -1 snails; 26.0 ± 1.4 s for Day 1 snails; and 30.2 ± 1.7 s for Day 5 snails. The number of snails was 40 each. There was no difference in the time of emergence following the 3-s electric shock in the different groups ($F_{2,117}$ = 1.936, P > 0.05). Therefore, the US was similarly perceived in Day -1, Day 1 and Day 5 snails.

Overwhelmed memory in severely food-deprived snails

We have suggested snails engage in the 'necessity knows no laws' concept in Introduction section. The concept of 'necessity knows no laws' has been in the literature since the time of Publilius Syrus (*ca.* 100 BC) and has been considered as an English proverb since the 1550's (Oxford English Dictionary). It basically means, "during a famine a very honest person may break the law to feed their children". Or "if one is desperate one may have to do illegal things". Thus, if snails are fed, they will adhere to the law (i.e., aversive food conditioning) and not eat. However, if sufficiently food-deprived (i.e., being desperate), they will break the law and eat.

To test our hypothesis that Day 5 snails indeed learn and form LTM but the behavioral phenotype of LTM is occluded by the behavioral choice mechanism underlying the 'necessity knows no law' concept, we trained 4 different cohorts of naive Day 5 snails using the 10 mmol l^{-1} sucrose solution as the CS and the 3-s electric shock as the US (Fig. 2A). We found in the first cohort (Group A in Fig. 2B; the number of snails was 20 in each group) that following 20 pairings of the CS and US in the 10 min posttest CS application, the feeding was elicited, indicating that CTA was not observed (Sugai et al., 2007; Mita et al., 2014a,b).

If the behavioral phenotype of LTM in Day 5 trained snails is overwhelmed by hunger when tested with the 10 mmol l^{-1} sucrose CS then possibly the LTM phenotype would re-emerge in these snails following subsequent access to food. To test this possibility, we trained a second naive cohort of Day 5 snails (Group B in Fig. 2C) using 10 mmol l^{-1} sucrose as the CS and the 3-s electric shock as the US. However, following 20 pairings of the CS and US, we allowed these Day 5 snails to have *ad libitum* access to food for 7 days (i.e., 7 days later after training as Day 5 snails). When we tested these snails for CTA memory (i.e., on Day 12), they did not exhibit memory (Fig. 2C). Thus, if memory had been formed in Day 5 snails and occluded by hunger, allowing subsequent feeding to alleviate hunger in these snails did not bring about the emergence of LTM.

However, the both facts that food deprivation produces a very specific behavioral state,

which is considered to be a context (Palmer & Kristan, 2011; Dyakonova, 2014), and that memory is context-dependent in aerial respiratory behavior of *Lymnaea* (Haney & Lukowiak, 2001) suggest that snails may only recall CTA memory when they are in a similar context in which they learned and formed CTA-LTM. Here, the context would be the state associated with food deprivation. Thus, we trained another Day 5 group of snails (Group C in Fig. 2D). In this cohort following the 7-day *ad libitum* access to food, snails were then subjected to a 1-day food deprivation period before receiving the CS posttest (i.e., Day 13 snails). These snails exhibited LTM for CTA in comparison with the controls at the posttests ($P < 0.05$, Fig. 2D). This CTA-LTM was observed at 10 min, 1 h, 1 day and 1 week posttests.

As a final test of the hypothesis, we trained a fourth cohort of Day 5 snails (Group D in Fig. 2E). In these snails we did not test for LTM following the interposed 1 day of food deprivation, but only tested for LTM after the 7 days of food access. In these snails LTM was not observed (Fig. 2E). The significant difference between this cohort of snails and the previous one is that these snails did not have a memory test following the food deprivation context.

We tried three further experiments. In the first group, Day 5 snails were trained, then given the '7-day' *ad libitum* access to food, followed by a second 5-day food deprivation period before receiving the CS posttest. In the second group, Day 5 snails were trained, then given the '1-day' *ad libitum* access to food, followed by a second 5-day food deprivation period and then the CS posttest. However, we found that as a result of the second 5 days of food deprivation too many snails were in poor physical condition. In any case, these data showed that prolonged food deprivation in *Lymnaea* clearly results in an overly stressed state. In the third group, we performed one more experiment to further test our hypothesis. Day 1 snails were trained, then given the 7-day *ad libitum* access to food, followed by a 5-day food deprivation period before receiving the CS posttest. Although the snails acquired CTA following training, the snails failed to show CTA-LTM after 5-day food deprivation (10.6 ± 1.2 bites/min for taste-aversion trained snails; 12.1 ± 1.0 bites/min for backward-conditioned snails; 13.6 ± 0.6 bites/min for naive snails; $F_{5,114} = 2.054$, $P > 0.05$, n = 20 each). Thus again,

severe food deprivation blocks the ability to express LTM.

Insulin effects in severely food-deprived snails

Finally, we attempted to use an insulin receptor antibody to examine the effect of insulin on CTA-LTM of the Group C snails (i.e., Day 13 snails). We have previously showed that the injection of an insulin receptor antibody to the isolated central nervous system (CNS) blocked the long-term enhancement of a synaptic connections in the feeding circuitry underlying CTA-LTM (Murakami et al., 2013; Mita et al., 2014a,b). We therefore trained Day 5 snails by a taste aversion protocol. Following 20 CS-US pairings, we injected the insulin receptor antibody into the abdominal cavity of the snails, and allowed them to have access *ad libitum* to food for 7 days. Then these snails were subjected to a 1-day food deprivation period before receiving the posttest. Unlike the Day 13 snails shown in Fig. 2D, these Day 13 snails that received the insulin receptor antibody injection did not exhibit CTA-LTM (Fig. 3A). As a control experiment, we injected IgG instead of the insulin receptor antibody into Day 13 snails and in these control snails CTA-LTM was observed (Fig. 3B). This CTA-LTM was observed at 10 min, 1 h, 1 day and 1 week posttests.

DISCUSSION

In previous studies, we concluded that LTM was not present in snails subjected to prolonged food deprivation (i.e., Day 5 snails) (Sugai et al., 2007; Mita et al., 2014a,b). However, as shown here that conclusion was not entirely correct. In this present study we showed that in Day 13 snails (i.e., snails subjected to the initial 5-day food deprivation, but then allowed 7-day access to food followed by 1-day food-deprivation) LTM was present. This was in contrast to Day 12 snails which did not exhibit LTM. There were two obvious differences between Day 12 and Day 13 snails. First, there was the factor of context in which LTM was tested. That is, the expression of the memory was not seen if the context was different from the training context (i.e., a food-deprived state). Second, there was the problem of conflict resolution between satisfying the

need 'to eat vs. not to eat', because there was a memory resulting from the training procedure.

LTM expression in *Lymnaea* has been shown to be context-dependent (Haney & Lukowiak, 2001). Thus, following training in 5-day food-deprived snails, allowing snails to have access to food for 7 days, and then triggering their memory by depriving them of food (i.e., recreating the context in which they were trained) allows memory expression to occur. The CTA-LTM was present in Day 5 trained snails when tested in the context (i.e., a food-deprived state) in which the memory was originally made in.

Our data clearly showed that a one-day period of food deprivation was necessary for CTA-LTM but that a period of 5 days of food deprivation obscured the memory. That is, following the 'necessity knows no law' concept, the memory was not seen in these snails. Presently we do not understand how food deprivation brings about these changes in memory. However, we do know that food deprivation causes a number of physiological changes in snails. For example, serotonin (5-hydroxytryptamine: 5-HT), a multimodal transmitter, controls both feeding and cardiovascular behaviors in snails (Kemenes et al., 1989; Buckett et al., 1990; Hatakeyama et al., 1999; Yamanaka et al., 2000; Kawai et al., 2011). Recently, Yamagishi and colleagues found that food deprivation significantly alters heart rate activity in *Lymnaea* (Yamagishi et al., 2015). They found that heart rate in food-satiated snails (i.e., Day -1 snails) is significantly higher than that in food-deprived snails (i.e., Day 5 snails). Further in food-deprived but not in food-satiated snails injection of 5-HT boosted heart rate. These data are consistent with the hypothesis that the exogenously triggered increase in 5-HT mimics the change from a food deprivation to a food satiation state normally achieved by direct ingestion of food.

In the same vein, Dyakonova and colleagues demonstrated that 5-HTergic neurons directly sense the concentration of hemolymph glucose (Dyakonova et al., 2015a,b).They showed that in vitro 5-HTergic neurons controlling locomotion alters biophysical properties in response to exogenous glucose application, resulting in a decreased resting membrane potential and a concomitant decreased spontaneous firing

rate. Additionally, the exogenous application of glucose to the isolated pedal ganglia causes a decrease in excitatory input to those 5-HTergic neurons. These data are consistent with previous data showing that food deprivation increases the activity and synaptic inputs of certain select neurons in the pedal ganglia (Dyakonova & Sakharov, 2001a,b; Chistopolsky & Sakharov, 2003; Dyakonova, 2014).

It is also becoming evident that the neurochemical control of behavior during food deprivation consists of multi-components. A change in 5-HT, dopamine or glucose content alone during starvation cannot explain various behavioral changes that are observed during food deprivation. It is likely that the chemical parameters change at different stages of starvation and they are different for different behaviors that depend upon a hunger state.

As can be seen in Results, when we used 10 mmol l^{-1} sucrose as the CS, the number of bites in snails elicited by this CS did not reflect how long snails were food-deprived. That is, the CS elicited a similar feeding response in all groups of snails tested. However, it is correct that the number of bites elicited by the CS (sucrose) depends on the concentration of sucrose (Ito et al., 2015). The higher concentration of sucrose was applied, the greater number of bites was observed. However, even a higher concentration of sucrose was used (100 mmol l^{-1}) as the CS, the feeding response elicited was not significantly different among snails with different durations of food deprivation (Ito et al., 2015).

Previously we found that some isoforms of molluscan insulin-related peptides were up-regulated following CTA-LTM (Azami et al., 2006). More recently, Murakami et al. (2013) examined the effects of insulin on CTA-LTM, and found that insulin acted at a synaptic connection in the neural circuit underlying CTA-LTM. We then further hypothesized that the change in hemolymph glucose concentration was caused by a rise in the insulin concentration (insulin spike) triggered by the CS (sucrose to the lips) during the course of taste aversion training (Mita et al., 2014a, b). These data, together with the present results, suggest that the modest food deprivation for memory expression may cause the insulin spike in the CNS. We speculate that such an insulin

spike does not occur in the backward-conditioned snails, This is based on our observation that when insulin was injected into the food-deprived snails, learning and CTA memory was improved (Mita et al., 2014b). Thus, the occurrence of an insulin spike correlates with the acquisition and retention of associative learning. In the near future, we will determine the trace amount of insulin concentration in the *Lymnaea* CNS (Watabe et al., 2014).

Stress alters many different aspects of learning, memory formation and its recall (Shors, 2004). Because stress has a broad definition, different stressors may have different biological significances and even opposite behavioral effects. In *Lymnaea*, it has been amply demonstrated that some stressors block LTM formation, while others enhance LTM formation (Lukowiak et al., 2014). It becomes even more complicated when multiple stressors are encountered. In some cases, it appears that the contribution of each stressor is additive while in other cases there are emergent properties when stressors are combined (Dalesman et al., 2013). Here we have shown for the first time in *Lymnaea* that different stress states resulting from different durations of food deprivation also alters the ability to express LTM.

In conclusion, snails can learn and exhibit CTA-LTM following 5-day food-deprivation period. However, the LTM phenotype is only observed in these snails if: (1) they are no longer food-deprived (i.e., they are tested for LTM following *ad libitum* access to food for an additional 7 days; and (2) they are in a one-day food-derived state. Thus, snails do not express the memory phenotype if they are extremely hungry (i.e., Day 5 snails) or if they have recovered from 5-day food deprivation but are tested in a food satiated state (i.e., Day 12 snails). Thus the expression of memory is both context dependent and may only be expressed following the resolution of the conflict between the homeostatic drive to eat vs. having a memory of learning not to eat. That is, snails solve this conflict by adhering to the ‘necessity knows no law’ concept.

MATERIALS AND METHODS

Snails

Specimens of *Lymnaea stagnalis* Linnaeus 1758 with an 18-23 mm shell obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam) were used in the present study. All snails were maintained in dechlorinated tap water (i.e., pond water) under a 12:12 light-dark cycle at 20^{o}C and fed *ad libitum* on turnip leaves (*Brassica rapa* var. *peruviridis*; Komatsuna [in Japanese]) and a commercially available product called Spiral Shell Food (a combination of seaweed, brewer's yeast and vitamins; Nisso, Saitama, Japan) every other day. *Lymnaea* exhibit good growth and reproduction under these conditions.

Taste aversion training (CTA) procedure

The basic procedure of taste aversion training was described previously (Fig. 1A; Wagatsuma et al., 2004; Ito et al., 2012, 2015). Briefly, all snails were first given a pretest in polystyrene petri dishes (diameter 35 mm). That is, we counted by visual inspection the number of feeding responses (i.e., bites; rasping movements of the buccal mass) elicited by the CS in the one-minute period after presentation of the CS. The size of mouth openings did not depend on the status of food deprivation. Thus, we only recorded the number of bites per min elicited by the CS. Following to the pretest, the conditioning and control procedures were all performed to the snails in the same petri dish as the pretest. In the taste aversion training procedure, we paired the CS (10 mmol l^{-1} sucrose solution) with the US (3-s electric shock; Takigami et al., 2013; Ito et al., 2015). The CS was rinsed out by distilled water and then followed by the US. The US period was set as 15 s, because following the 3-s electric shock a 12-s recovery time was needed to re-emerge the body from the shells (Ito et al., 2015). The electric shock was applied near the head in the distilled water. Snails received 20 paired presentations of the CS-US. Controls included a backward-conditioned (US-CS) group and a naive group to validate associative learning. For the naive control group, only distilled water was applied to the lips instead of the CS and US. In the posttest sessions, snails were

again challenged with the CS, and the number of bites was recorded in the 1-min interval in distilled water after a 15-s application of the CS. In all experiments, after the 10 min posttest, snails were allowed *ad libitum* access to food. All tests were performed blindly.

Injection of insulin receptor antibody

To examine the effect of insulin on CTA-LTM, we used the mouse monoclonal [47-9] antibody to the insulin receptor alpha subunit (ab982, Abcam, Cambridge, UK), whose final concentration in the body was estimated as 2.56 μg ml^{-1} (17.5 nM). The details of the injection volume and injection part were described in the previous studies (Murakami et al., 2013). This antibody blocks the binding between insulin and insulin receptors (Soos et al., 1986; Taylor et al., 1987). The control experiments were performed by injection of IgG (4.4 μg ml^{-1} as its final concentration in *Lymnaea* saline; whole molecule, Jackson ImmonoResearch Laboratories, West Grove, PA; Murakami et al., 2013). *Lymnaea* saline consisted of 50 mmol l^{-1} NaCl, 1.6 mmol l^{-1} KCl, 2.0 mmol l^{-1} $MgCl_2$, 3.5 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} HEPES (pH 7.9).

Statistics

The data are expressed as the mean ± SEM. Significant differences at $P < 0.05$ were examined by one-way factorial ANOVA and post hoc Tukey test.

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Author contributions

E.I., V.D and K.L. designed the research; E.I., M.Y., D.H. and T.W. performed the experiments; E.I., D.H., T.W., Y.F., V.D. and K.L. analyzed the data; and E.I., D.H., T.W., Y.F., V.D., and K.L. wrote the paper.

Competing interests

The authors declare no competing interests.

References

Azami, S., Wagatsuma, A., Sadamoto, H., Hatakeyama, D., Usami, T., Fujie, M., Koyanagi, R., Azumi, K., Fujito, Y., Lukowiak, K. and Ito, E. (2006). Altered gene activity correlated with long-term memory formation of conditioned taste aversion in *Lymnaea*. *J. Neurosci. Res.* **84**, 1610-1620.

Benjamin, P. R., Staras, K. and Kemenes, G. (2000). A systems approach to the cellular analysis of associative learning in the pond snail *Lymnaea*. *Learn. Mem.* **7**, 124-131.

Buckett, K. J., Dockray, G. J., Osborne, N. N. and Benjamin, P. R. (1990). Pharmacology of the myogenic heart of the pond snail *Lymnaea stagnalis*. *J. Neurophysiol.* **63**, 1413-1425.

Chistopolsky, I. A. and Sakharov, D. A. (2003). Non-synaptic integration of neuron somata in the snail CNS. *Neurosci. Behav. Physiol.* **33**, 295-300.

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.

Dyakonova, V. E. (2014). Neurotransmitter mechanisms of context-dependent behavior. *Neurosci. Behav. Physiol*. **44**, 256-267.

Dyakonova, V. E. D. and Sakharov, D. A. (2001a). Firing of an isolated serotonergic neuron depends on the rate of neurotransmitter synthesis. *Doklady Biol. Sci.* **376**, 267-270.

Dyakonova, V. E. D. and Sakharov, D. A. (2001b). An isolated serotonergic neuron: The mechanism of excitation induced by enhanced synthesis of neurotransmitter. *Doklady Biol. Sci.* **378**, 694-696.

Dyakonova, V., Hernádi, L., Ito, E., Dyakonova, T., Zakharov, I. and Sakharov, D. (2015a). The activity of isolated snail neurons controlling locomotion is affected by glucose. *BIOPHYSICS* **11**, 55-60.

Dyakonova, V. E., Hernádi, L., Ito, E., Dyakonova, T. L., Chistopolsky, I. A., Zakharov, I. S. and Sakharov, D. A. (2015b) The activity of isolated neurons and the modulatory state of an isolated nervous system represent a recent behavioural state. *J. Exp. Biol.* in press.

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. and Ito, E. (1999). Three-dimensional reconstruction and mapping of serotonin-like immunoreactive neurons in the central nervous system of the pond snail, *Lymnaea stagnails*, with the confocal laser scanning microscope, *Bioimages* **7**, 1-12.

Ito, E., Kobayashi, S., Kojima, S., Sadamoto, H. and Hatakeyama, D. (1999). Associative learning in the pond snail, *Lymnaea stagnalis*. *Zool. Sci.* **16**, 711-723.

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.

Ito, E., Kojima, S., Lukowiak, K. and Sakakibara, M. (2013). From likes to dislikes: conditioned taste aversion in the pond snail *Lymnaea stagnalis*. *Can. J. Zool.* **91**, 405-412.

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

Kawai, R., Sunada, H., Horikoshi, T. and Sakakibara, M. (2004). Conditioned taste aversion with sucrose and tactile stimuli in the pond snail *Lymnaea stagnalis*. *Neurobiol. Learn. Mem.* **82**, 164-168.

Kawai, R., Kobayashi, S., Fujito, Y. and Ito E. (2011). Multiple subtypes of serotonin receptors in the feeding circuit of a pond snail. *Zool. Sci.* **28**, 517-525.

Kemenes, G., Elekes, K., Hiripi, L. and Benjamin, P. R. (1989). A comparison of four techniques for mapping the distribution of serotonin and serotonin-containing neurons in fixed and living ganglia of the snail, *Lymnaea*. *J. Neurocytol.* **18**, 193-208.

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.

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.

Mita, K., Okuta, A., Okada, R., Hatakeyama, D., Otsuka, E., Yamagishi, M., Morikawa, M., Naganuma, Y., Fujito, Y., Dyakonova, V., Lukowiak, K. and Ito, E. (2014a). What are the elements of motivation for acquisition of conditioned taste aversion? *Neurobiol. Learn. Mem.* **107**, 1-12.

Mita, K., Yamagishi, M., Fujito, Y., Lukowiak, K. and Ito, E. (2014b). An increase in insulin is important for the acquisition conditioned taste aversion in *Lymnaea*. *Neurobiol. Learn. Mem.* **116**, 132-138.

Murakami, J., Okada, R., Sadamoto, H., Kobayashi, S., Mita, K., Sakamoto, Y., Yamagishi, M., Hatakeyama, D., Otsuka, E., Okuta, A., Sunada, H., Takigami, S., Sakakibara, M., Fujito, Y., Awaji, M., Moriyama, S., Lukowiak, K. and Ito, E. (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.

Otsuka, E., Matsunaga, M., Okada, R., Yamagishi, M., Okuta, A., Lukowiak, K. and Ito, E. (2013). Increase in cyclic AMP concentration in a cerebral giant interneuron mimics part of a memory trace for conditioned taste aversion of the pond snail. *BIOPHYSICS* **9**, 161-166.

Palmer, C. R. and Kristan, W. B. Jr. (2011). Contextual modulation of behavioral choice. *Curr. Opin. Neurobiol.* **21**, 520-526.

Shors, T. J. (2004). Learning during stressful times. *Learn. Mem.* **11**, 137-144.

Soos, M. A., Siddle, K., Baron, M. D., Heward, J. M., Luzio, J. P., Bellatin, J. and Lennox, E. S. (1986). Monoclonal antibodies reacting with multiple epitopes on the human insulin receptor. *Biochem. J.* **235**, 199-208.

Sugai, R., Shiga, H., Azami, S., Watanabe, T., Sadamoto, H., Fujito, Y., Lukowiak, K. and Ito, E. (2006). Taste discrimination in conditioned taste aversion of the pond snail *Lymnaea stagnalis*. *J. Exp. Biol.* **209**, 826-833.

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.

Sunada, H., Takigami, S., Lukowiak, K. and Sakakibara, M. (2014). Electrophysiological characteristics of feeding-related neurons after taste

avoidance Pavlovian conditioning in *Lymnaea stagnalis*. *BIOPHYSICS* **10**, 121-133.

Takahashi, T., Takigami, S., Sunada, H., Lukowiak, K. and Sakakibara, M. (2013). Critical period of memory enhancement during taste avoidance conditioning in *Lymnaea stagnalis*. *PLoS One* **8**, e75276.

Takigami, S., Sunada, H., Lukowiak, K. and Sakakibara, M. (2013). High voltage with little current as an unconditional stimulus for taste avoidance conditioning in *Lymnaea stagnalis*. *Neurosci. Lett.* **555**, 149-153.

Takigami, S., Sunada, H., Lukowiak, K. and Sakakibara, M. (2014). Spaced taste avoidance conditioning in *Lymnaea*. *Neurobiol. Learn. Mem.* **107**, 79-86.

Taylor, R., Soos, M. A., Wells, A., Argyraki, M. and Siddle, K. (1987). Insulin-like and insulin-inhibitory effects of monoclonal antibodies for different epitopes on the human insulin receptor. *Biochem. J.* **242**, 123-129.

Wagatsuma, A., Sugai, R., Chono, K., Azami, S., Hatakeyama, D., Sadamoto, H. and Ito, E. (2004). The early snail acquires the learning. comparison of scores for conditioned taste aversion between morning and afternoon. *Acta Biol. Hung.* **55**, 149-155.

Watabe, S., Kodama, H., Kaneda, M., Morikawa, M., Nakaishi, K., Yoshimura, T., Iwai, A., Miura, T. and Ito, E. (2014). Ultrasensitive enzyme-linked immunosorbent assay (ELISA) of proteins by combination with the thio-NAD cycling method. *BIOPHYSICS* **10**, 49-54.

Yamagishi, M., Watanabe, T., Hatakeyama, D. and Ito, E. (2015). Effects of serotonin on the heartbeat of pond snails in a hunger state. *BIOPHYSICS* **11**, 1-5.

Yamanaka, M., Hatakeyama, D., Sadamoto, H., Kimura, T. and Ito, E. (2000). Development of key neurons for learning stimulates learning ability in *Lymnaea stagnalis*. *Neurosci. Lett.* **278**, 113-116.

Figures

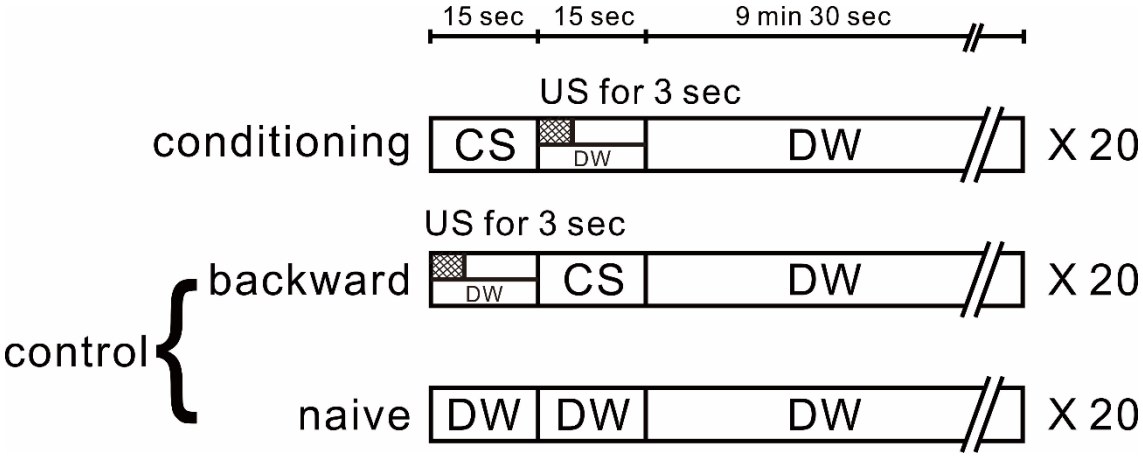

Fig. 1. Learning procedure of conditioned taste aversion in *Lymnaea*. All snails were first given a pretest. In this observation period (1 min), the number of feeding responses (i.e., bites/min) was counted in distilled water following a 15-s application of 10 mmol l^{-1} sucrose (the CS) to the lips of the snail. For taste aversion training, the 10 mmol l^{-1} sucrose CS was paired with the 3-s high voltage electric shock (the US). Following the 3-s electric shock, a 12-s recovery period was required in the US period. The inter-stimulus interval was 15 s between the onset of the CS and US. A 10-min inter-trial interval was interposed between each pairing of the CS-US. Snails received 20 paired CS-US trials on a single day. Controls included a backward-conditioned (US-CS) group and a naive group to validate associative learning. For the naive control group, only distilled water was applied to the lips instead of the CS and US. In the posttest sessions, snails were again challenged with the CS, and the number of bites was recorded in the 1-min interval in distilled water after a 15-s application of the CS. AF: The snails were fed *ad libitum* on turnip leaves and Spiral Shell Food; FD: The snails were food-deprived; TAT: The snails were taste-aversion trained; PT: The snails were posttested.

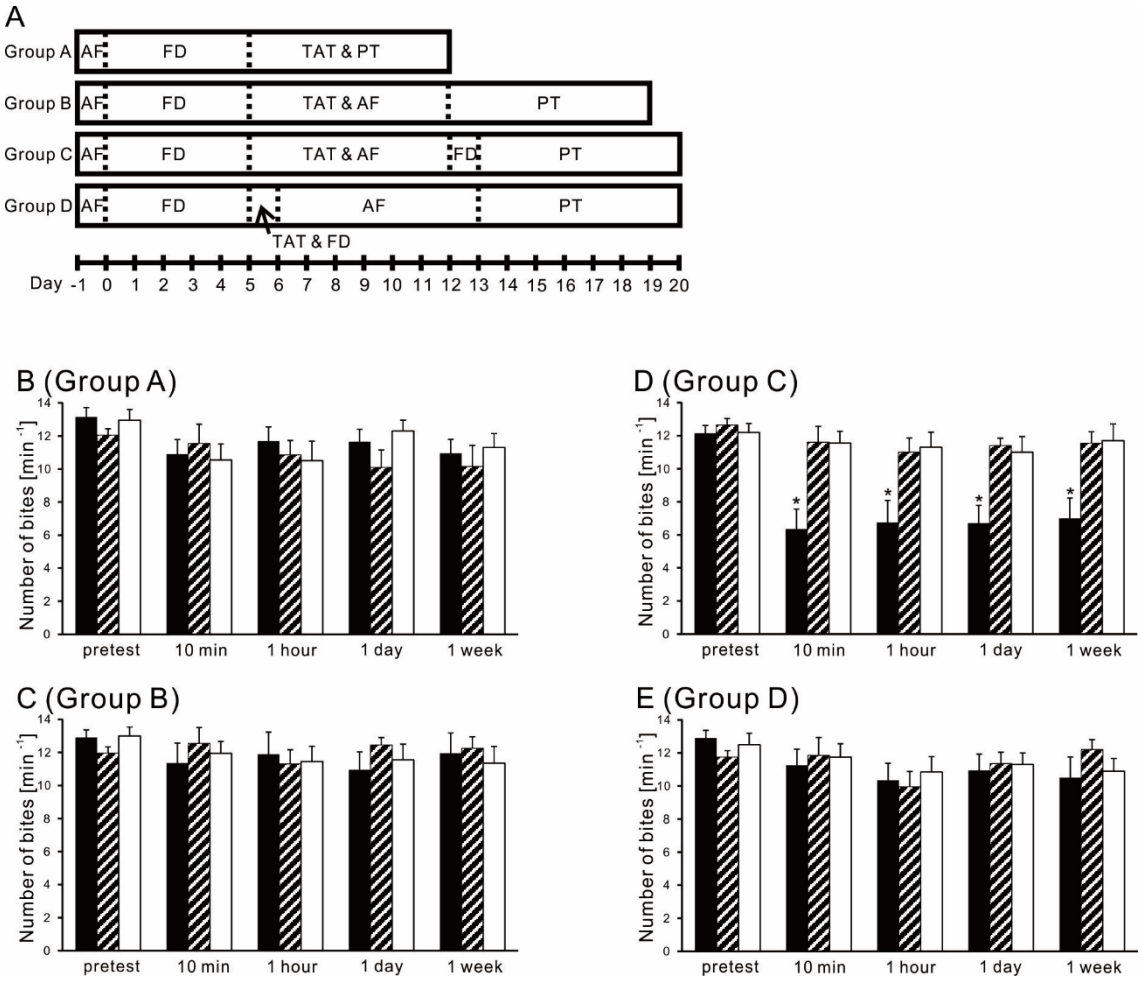

Fig. 2. Memory formation in 5-day severely food-deprived snails. (A) Timing of taste aversion training and posttest. We prepared 4 cohorts (Groups A - D) of Day 5 snails; that is, severely food-deprived snails. The day when snails began food deprivation was designated Day 0. AF: The snails were fed *ad libitum* on turnip leaves and Spiral Shell Food. FD: The snails were food-deprived. TAT: The snails were taste-aversion trained. PT: The snails were posttested. (B) Numbers of bites/min elicited by the CS (a 10 mmol l^{-1} sucrose solution) in the pretest session and the posttest sessions for Group A. Using a 15-s application of CS (a 10 mmol l^{-1} sucrose solution) and a 3-s application of US (a high voltage electric shock), we prepared taste-aversion trained (black bars), backward-conditioned (hatched bars) and naive control snails (white bars) with 20 pairings of CS-US. These snails were severely food-deprived Day 5 snails. No memory retention was found ($F_{14,285}$ = 1.105, P > 0.05, n = 20 each). (C) Numbers of bites/min elicited by the CS in the pretest session and the posttest sessions for Group B.

No memory retention was found ($F_{14,285} = 0.461$, $P > 0.05$, n = 20 each). (D) Numbers of bites/min elicited by the CS in the pretest session and the posttest sessions for Group C; that is, the snails were food-deprived again for one additional day. The feeding response to the CS was significantly reduced ($F_{14,285} = 5.434$, $P < 0.01$, n = 20 each) at the posttest, compared to those observed for the backward-conditioned and naive control snails. This aversive behavior was consolidated to CTA-LTM that was recorded at the posttests at 1 h, 1 day and 1 week (*$P < 0.05$). (E) Numbers of bites/min elicited by the CS in the pretest session and the posttest sessions for Group D. No memory retention was found ($F_{14,285} = 0.917$, $P > 0.05$, n = 20 each).

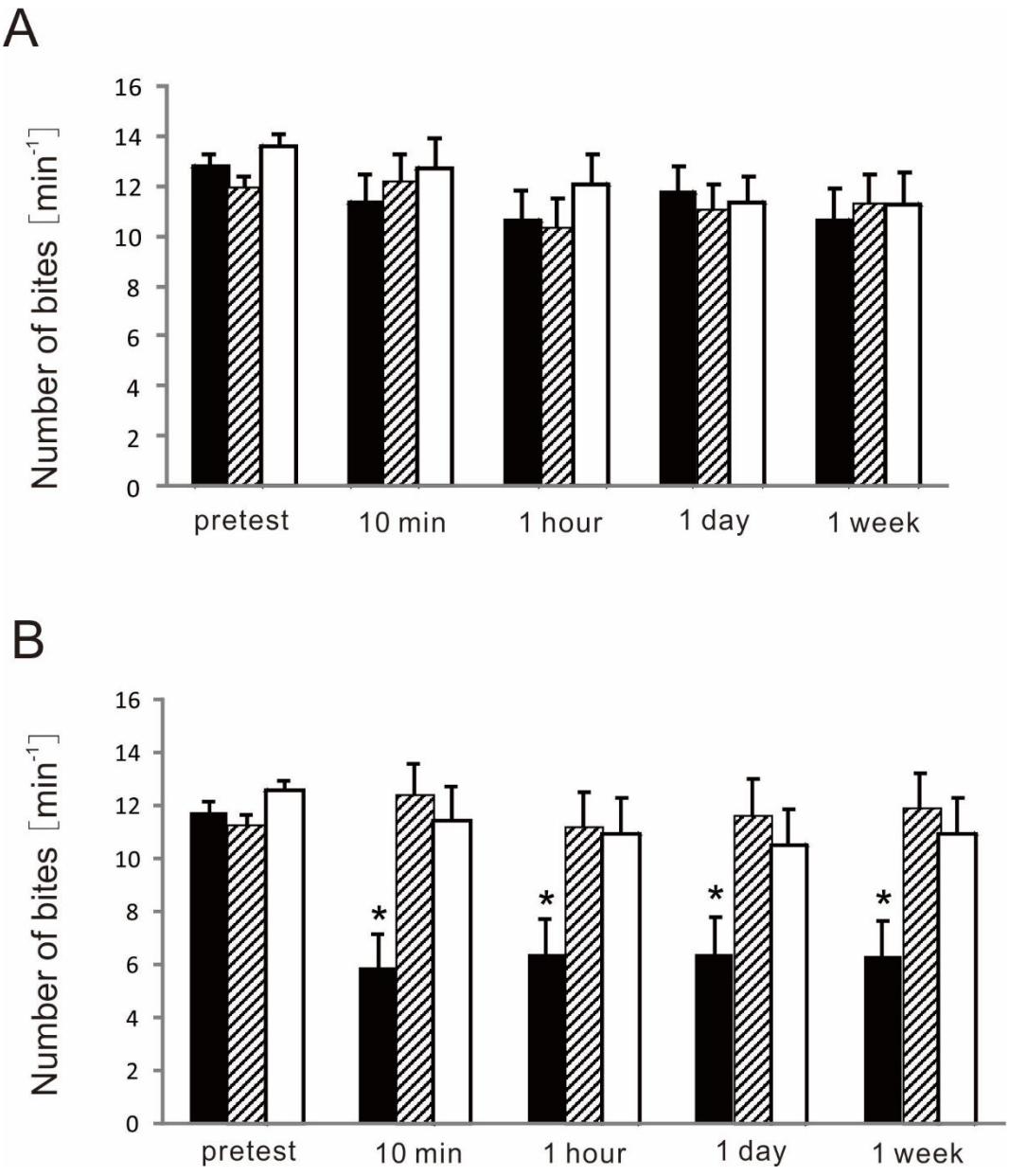

Fig. 3. Effect of insulin receptor antibody on appearance of overwhelmed memory. (A) Numbers of bites/min elicited by the CS in the pretest session and the posttest sessions for snails that were injected by an insulin receptor antibody. The other conditions were the same as those for Group C in Fig. 2D. No memory retention was found ($F_{14,285} = 1.077$, $P > 0.05$, n = 20 each). (B) Control experiments for (A) using IgG instead of insulin receptor antibody. The aversive behavior was again observed like Group C in Fig. 2D ($F_{14,285} = 6.083$, $P < 0.01$, n = 20 each). * indicates $P < 0.05$.