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RESEARCH ARTICLE

Memory block: a consequence of conflict resolution

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ABSTRACT

Food deprivation for 1 day in the pond snail *Lymnaea stagnalis* before aversive classical conditioning results in optimal conditioned taste aversion (CTA) and long-term memory (LTM) formation, whereas 5-day food deprivation before training does not. We hypothesize 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 is overpowered by severe food deprivation. Moreover, CTA-LTM was context dependent and was 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, an appetitive stimulus, such as sucrose [the conditioned stimulus (CS)], is repeatedly paired with an aversive stimulus, such as an 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, food deprivation for 1 day results in the best learning and memory. Most interesting to us was the finding that food deprivation for 5 days before training results 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 or 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 and Lukowiak, 2001) plays an important role in the lack of 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 were fed *ad libitum*. They were not food-deprived. Day 1 and day 5 snails were food-deprived for 1 day or 5 days, respectively. After day 5, snails had *ad libitum* (turnip leaves and Spiral Shell food). Day 12 snails had *ad libitum* access to food for an additional 7 days. Day 13 snails were food-deprived for an additional 1 day.

Snails were deemed to be healthy under all the above conditions because they continued to exhibit normal homeostatic behaviors such as aerial respiration, copulation and egg-laying. 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, but we did not find any significant difference in the 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).

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^{-1} for day −1 snails; 13.0±0.4 bites min^{-1} for day 1 snails; and 11.9±0.2

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List of abbreviations

AF	*ad libitum* feeding on turnip leaves and Spiral Shell Food
CS	conditioned stimulus
CTA	conditioned taste aversion
FD	food deprivation
LTM	long-term memory
PT	post-tested
TAT	taste-aversion training
US	unconditioned stimulus
US–CS	backward conditioning

bites min^{-1} 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 stressed 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 for 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 for each group. 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.

Overpowered memory in severely food-deprived snails

We have suggested that snails engage in the 'necessity knows no laws' concept in the Introduction. This concept has been discussed in the literature since the time of Publilius Syrus (ca. 100 BC) and has been considered as an English proverb since the 1550s (Oxford English Dictionary). It basically means that 'during a famine a very honest person may break the law to feed their children' or 'if one is

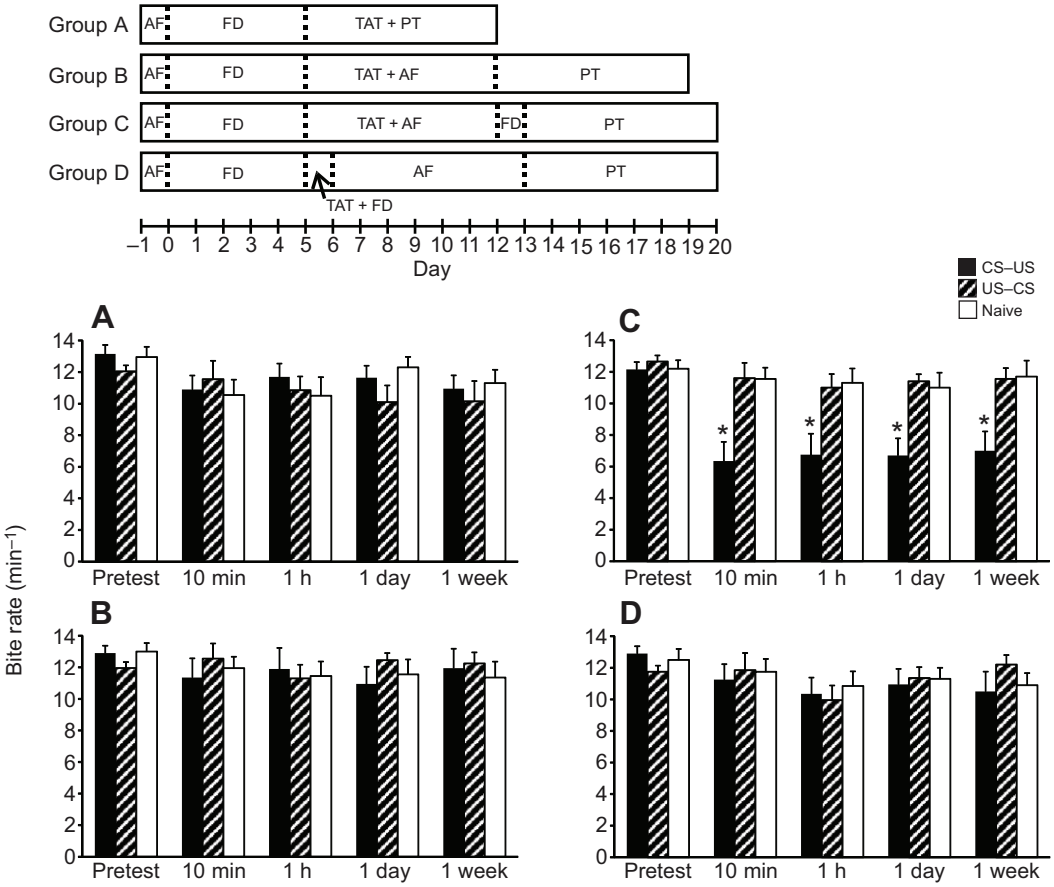

Fig. 1. Memory formation in 5-day severely food-deprived *Lymnaea* snails. Top panel shows timing of taste aversion training and post-test. We prepared four cohorts (groups A–D) of day 5 snails; that is, severely food-deprived snails. The day when snails began food deprivation was designated day 0. AF, snails were fed *ad libitum* on turnip leaves and Spiral Shell Food; FD, snails were food-deprived; TAT, snails were taste-aversion trained; PT, snails were post-tested. (A) Numbers of bites min^{-1} elicited by the CS (a 10 mmol l^{-1} sucrose solution) in the pretest session and the post-test 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 (CS–US), backward-conditioned (US–CS) and naive control snails 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). (B) Numbers of bites min^{-1} elicited by the CS in the pretest session and the post-test sessions for Group B. No memory retention was found ($F_{14,285}$=0.461, P>0.05, N=20 each). (C) Numbers of bites min^{-1} elicited by the CS in the pretest session and the post-test 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 post-test, compared with those observed for the backward-conditioned and naive control snails. This aversive behavior was consolidated to CTA-LTM that was recorded at the post-tests at 1 h, 1 day and 1 week (*P<0.05). (D) Numbers of bites min^{-1} elicited by the CS in the pretest session and the post-test sessions for Group D. No memory retention was found ($F_{14,285}$=0.917, P>0.05, N=20 each).

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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 four different cohorts of naive day 5 snails using 10 mmol l^{-1} sucrose solution as the CS and 3 s electric shock as the US (Fig. 1). We found in the first cohort (group A, Fig. 1A; N= 20 for each group) that following 20 pairings of the CS and US in the 10 min post-test 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 overpowered 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, Fig. 1B) using 10 mmol l^{-1} sucrose as the CS and the 3-s electric shock as the US. Following 20 pairings of the CS and US, we allowed then these day 5 snails to have *ad libitum* access to food for 7 days (i.e. 7 days 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. 1B). 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 fact that food deprivation produces a very specific behavioral state, which is considered to be a context (Palmer and Kristan, 2011; D'yakonova, 2014) and since memory is context dependent in the aerial respiratory behavior of *Lymnaea* (Haney and Lukowiak, 2001), 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, Fig. 1C). In this cohort, following the *ad libitum* access to food for 7 days, snails were then subjected to a 1-day food deprivation period before receiving the CS post-test (i.e. day 13 snails). These snails exhibited LTM for CTA in comparison with the controls at the post-tests (P<0.05, Fig. 1C). This CTA-LTM was observed at 10 min, 1 h, 1 day and 1 week post-tests.

As a final test of the hypothesis, we trained a fourth cohort of day 5 snails (group D, in Fig. 1D). In these snails, we did not test for LTM following the interposed 1 day of food deprivation, but only tested for LTM after 7 days of access to food. In these snails, LTM was not observed (Fig. 1D). 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 7 days of *ad libitum* access to food, followed by a second 5-day food deprivation period before receiving the CS post-test. In the second group, day 5 snails were trained, then given 1 day of *ad libitum* access to food, followed by a second 5-day food deprivation period and then the CS post-test. We found that as a result of the second period of 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 7 days of *ad libitum* access to food, followed by a 5-day food deprivation period before receiving the CS post-test. Although the snails acquired CTA following training, these snails failed to show CTA-LTM after 5 days of food deprivation (10.6±1.2 bites min^{-1} for taste-aversion trained snails; 12.1±1.0 bites min^{-1} for backward-conditioned snails; 13.6±0.6 bites min^{-1} 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 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 post-test. Unlike the day 13 snails shown in Fig. 1C, these day 13 snails that received the insulin receptor antibody injection did not exhibit CTA-LTM (Fig. 2A). 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. 2B). This CTA-LTM was observed at 10 min, 1 h, 1 day and 1 week post-tests.

DISCUSSION

In previous studies, we concluded that LTM was not present in snails subjected to prolonged food deprivation (i.e. day 5 snails)

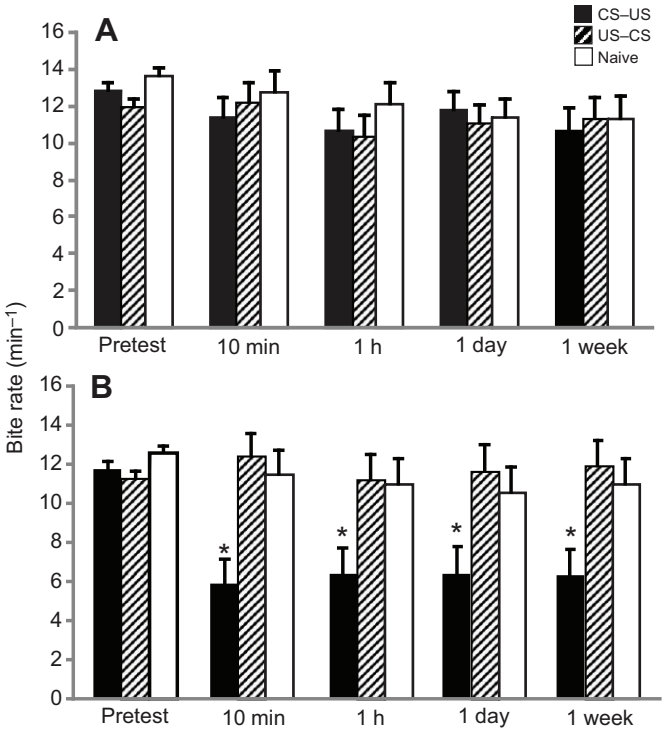

Fig. 2. Effect of insulin receptor antibody on memory in *Lymnaea*. (A) Numbers of bites min^{-1} elicited by the CS in the pretest session and the post-test sessions (10 min to 1 week) for snails that were injected with an insulin receptor antibody. The other conditions were the same as those for Group C in Fig. 1C. 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 as in Group C in Fig. 1C ($F_{14,285}$=6.083, P<0.01, N=20 each). *P<0.05.

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(Sugai et al., 2007; Mita et al., 2014a,b). However, as shown here, that conclusion was not entirely correct. In this present study, we show that in day 13 snails (i.e. snails subjected to the initial 5-day food deprivation, but then allowed access to food for 7 days, 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 the context in which LTM was tested. That is, 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 versus 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 and 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.

Our data clearly showed that a 1 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 and Ito, 1999; Yamanaka et al., 2000; Kawai et al., 2011). Recently, Yamagishi and colleagues found that food deprivation significantly alters heart rate 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 that is 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 show 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 and Sakharov, 2001a,b; Chistopol'skii and Sakharov, 2003; D'yakonova, 2014).

It is also becoming evident that the neurochemical control of behavior during food deprivation consists of multiple 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 our results, when we used 10 mmol l^{-1} sucrose as the CS, the number of bites elicited in snails 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, Ito et al. (2015) showed that the number of bites elicited by the CS (sucrose) depends on the concentration of sucrose. The higher the concentration of sucrose that was applied, the greater the number of bites observed. Even when a higher concentration (100 mmol l^{-1}) of sucrose was used 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 alter the ability to express LTM.

In conclusion, snails can learn and exhibit CTA-LTM following a 5-day food-deprivation period. 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 1-day food-deprived 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 versus 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 h:12 h light:dark cycle at 20°C and fed *ad libitum* on turnip leaves (*Brassica rapa*

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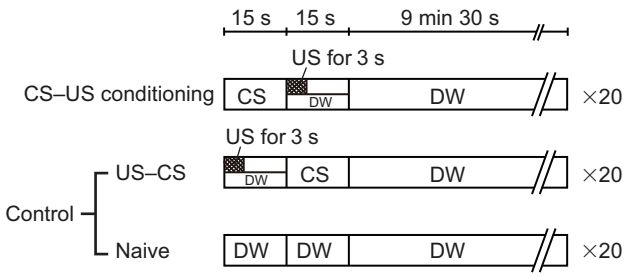

Fig. 3. Learning procedure of conditioned taste aversion in *Lymnaea*. All snails were first given a pretest. In this observation period (1 min), the number of feeding responses (i.e. bites min^{-1}) was counted in distilled water (DW) following a 15 s application of 10 mmol l^{-1} sucrose (the CS) to 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 post-test 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.

var. *peruviridis* known as Komatsuna) 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. 3; 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 1 min 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 the pretest, the conditioning and control procedures were all performed on 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 with distilled water and then followed by the US. The US period was set at 15 s, because following the 3 s electric shock, a 12 s recovery time was needed for the body to re-emerge 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 post-test 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 post-test, 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 antibody to the insulin receptor α-subunit (ab982, Abcam, Cambridge, UK), whose final concentration in the body was estimated as 2.56 μg ml^{-1} (17.5 nmol l^{-1}). The details of the injection volume and injection procedure were described previously (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, USA; 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

Data are expressed as means±s.e.m. Significant differences at $P<0.05$ were examined by one-way factorial ANOVA and *post hoc* Tukey test.

Competing interests

The authors declare no competing or financial interests.

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.

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