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What are the elements of motivation for acquisition of conditioned taste aversion?

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ABSTRACT

The pond snail *Lymnaea stagnalis* is capable of being classically conditioned to avoid food and to consolidate this aversion into a long-term memory (LTM). Previous studies have shown that the length of food deprivation is important for both the acquisition of taste aversion and its consolidation into LTM, which is referred to as conditioned taste aversion (CTA). Here we tested the hypothesis that the hemolymph glucose concentration is an important factor in the learning and memory of CTA. One-day food deprivation resulted in the best learning and memory, whereas more prolonged food deprivation had diminishing effects. Five-day food deprivation resulted in snails incapable of learning or remembering. During this food deprivation period, the hemolymph glucose concentration decreased. If snails were fed for 2 days following the 5-day food deprivation, their glucose levels increased significantly and they exhibited both learning and memory, but neither learning nor memory was as good as with the 1-day food-deprived snails. Injection of the snails with insulin to reduce glucose levels resulted in better learning and memory. Insulin is also known to cause a long-term enhancement of synaptic transmission between the feeding-related neurons. On the other hand, injection of glucose into 5-day food-deprived snails did not alter their inability to learn and remember. However, if these snails were fed on sucrose for 3 min, they then exhibited learning and memory formation. Our data suggest that hemolymph glucose concentration is an important factor in motivating acquisition of CTA in *Lymnaea* and that the action of insulin in the brain and the feeding behavior are also important factors.

1. Introduction

The pond snail *Lymnaea stagnalis* can be both classically and operantly conditioned and following the acquisition of learning forms memory. Using a proper training procedure, snails are able to consolidate the learning into long-term memory (LTM) (Benjamin, Kemenes, & Kemenes, 2008; Lukowiak et al., 2010; Sakakibara, 2006; Silverman-Gavrila, Senzel, Charlton, & Feng, 2011). We have focused our attention on the ability of *Lymnaea* to acquire taste aversion and to consolidate it into LTM. This is referred to as conditioned taste aversion (CTA) (Ito, Kobayashi, Kojima, Sadamoto, & Hatakeyama, 1999; Ito, Kojima, Lukowiak, & Sakakibara, 2013; Ito et al., 2012; Kawai, Sunada, Horikoshi, & Sakakibara, 2004; Kita et al., 2011; Takigami et al., 2013). The training procedure used to produce CTA in *Lymnaea* consists of an appetitive stimulus (i.e., sucrose) that is used as the conditioned stimulus (CS) and an aversive stimulus (i.e., KCl) that is used as the unconditioned stimulus (US). Application of the CS to the lips increases the feeding response in snails; whereas application of the US causes snails to withdraw into their shell and terminate feeding. In the taste aversion training procedure, the CS is paired with the US. After repeated temporal contingent presentations of the CS and US, the CS no longer elicits the feeding response, and this taste aversion persists for more than a month (Kojima, Yamanaka, Fujito, & Ito, 1996).

Previous studies using a one-trial training procedure for taste aversion learning demonstrated that a 1-day food deprivation period before training produced better and more consistent results (Sugai et al., 2007). A possible explanation for this observation is that the snails are more motivated to learn after food deprivation.

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Motivation certainly plays an important role in learning (Klahr, Zimmerman, & Jirout, 2011; Pompilio, Kacelnik, & Behmer, 2006; Walters, Carew, & Kandel, 1981). The 1-day food deprivation did not alter behaviors such as egg laying or aerial respiration, and did not result in any change in the general health of snails, such as evidenced by a change in mortality rate. However, more is not better always, because food deprivation for 5 days blocked taste aversion learning and its consolidation into LTM (Sugai et al., 2007). The 5-day food deprivation period might decrease the motivation for learning as the snail struggles to devise a survival strategy (Matsuo, Hitomi, Watanabe, & Kirino, 2002). We hypothesized here that the level of motivation in the snails might be correlated with the hemolymph glucose concentration, which may be controlled by a 'spike' in insulin secretion triggered by the CS. We therefore examined the relationship among the duration of the food deprivation period, the learning and memory-forming ability as assessed by the number of good vs. poor performers based on the definitions of Sugai et al., 2007, and the hemolymph glucose concentration in *Lymnaea* CTA.

The basis of our insulin hypothesis was our previous observation that molluscan insulin-related peptides (MIPs) were up-regulated in snails exhibiting CTA-LTM (Azami et al., 2006). We more recently showed at the electrophysiological level that the application of MIPs caused a long-term enhancement of synaptic transmission between the cerebral giant cell (CGC, a key interneuron for CTA) and the buccal B1 motor neuron (Murakami, Okada, Sadamoto, et al., 2013). This synaptic change has been hypothesized to play a key role in the CTA-LTM consolidation process. In further support of this hypothesis, we found that application of an insulin receptor antibody to the *Lymnaea* central nervous system (CNS) blocked the long-term enhancement of synaptic transmission. Finally, injection of the antibody into *Lymnaea* inhibited LTM formation (Murakami, Okada, Sadamoto, et al., 2013).

Therefore, we have to take into consideration the role played by glucose and insulin in CTA-LTM formation. Insulin secretion in the snail not only controls glucose concentration but also alters synaptic plasticity. In the present study, therefore, we examined taste aversion learning and LTM formation by observing the direct effects of changes in glucose concentration on the number of good vs. poor learning and memory performers. These experiments included various lengths of food deprivation, injection of glucose or insulin, and ingestion of sucrose. Our results suggest a strong correlation between the motivation of *Lymnaea* to acquire CTA and the hemolymph glucose concentration, and indicate that this relation may be controlled by insulin secretion.

2. Materials and methods

2.1. Snails

Specimens of *Lymnaea stagnalis* (L.) with an 18–23 mm shell were obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam). All snails were maintained in dechlorinated tapwater (i.e., pond water) under a 12:12 light–dark cycle at 20 °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 feeding conditions.

2.2. Taste aversion training procedure

All snails were first given a pretest in polystyrene Petri dishes (diameter 35 mm) (Sugai et al., 2006). In this observation period (1 min), the number of feeding responses (i.e., bites; rasping movements of the buccal mass) were counted in distilled water following a 15-s application of 10 mM sucrose (the CS) to the lips of the snail. In the taste aversion training procedure, we paired the 10 mM sucrose CS with the 10 mM KCl US. The duration of both the CS and the US was 15 s, with an inter-stimulus interval of 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 10 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. All tests were performed blindly. The behavioral experiments were performed in the morning, because the learning scores are better in the morning than at other times (Wagatsuma et al., 2004).

Based on our previous findings (Sugai et al., 2007), we set a performance boundary in the learning and memory test sessions to distinguish between good and poor performers. A snail possessing good learning and LTM (i.e., a good performer) is expected not to open its mouth following presentation of the CS. However, some snails open their mouths by chance (i.e., spontaneously) in the absence of any delivered stimulus (Kojima et al., 1996). Such spontaneous openings occur at a rate of about one per min. Thus, we defined a good performer as a snail that made 0–1 bites/min during the posttest session in response to presentation of the CS. Poor performers were thus defined as snails that made $\geqslant$2 bites/min in response to the CS during the posttest session. We plotted the percentage of good vs. poor performers for the various cohorts of snails subjected to food deprivation and/or experimental manipulations (e.g., injection of insulin) to better show the effect of the various manipulations on learning and memory.

2.3. Food deprivation status

Food deprivation status was defined in the following manner. The day when snails began food deprivation was designated Day 0.

Day −1 snails: The snails were fed *ad libitum* on turnip leaves and Spiral Shell Food.
Day 1 snails: The snails were food-deprived for 1 day.
Day 5 snails: The snails were food-deprived for 5 days.

After Day 5, snails were given *ad libitum* access to turnip leaves and Spiral Shell Food.

Day 7 snails: Snails had *ad libitum* access to food for 2 days.
Day 12 snails: Snails had *ad libitum* access to food for additional 5 days.

2.4. Hemolymph glucose concentration

To measure the hemolymph glucose concentration, the pond water surrounding the snail was blotted up with absorbent paper. The snail was then given a strong poke with a pipette, causing it to immediately retract into its shell and expel hemolymph through its renal pore. The collected hemolymph was heated at 95 °C for 10 min and concentrated at 11600 rpm for 10 min. The hemolymph glucose concentration was measured using either an Amplex Red glucose/glucose oxidase assay kit (Molecular Probes, Eugene, OR, USA) or a mutarotase-glucose oxidase assay kit (Glucose C2; Wako, Osaka, Japan). The measurements were performed according to the manufacturer's manual. The Amplex Red reagent is 10-acetyl-3,7-dihidroxyphenoxazine. When using the Amplex