THE EARLY SNAIL ACQUIRES THE LEARNING. COMPARISON OF SCORES FOR CONDITIONED TASTE AVERSION BETWEEN MORNING AND AFTERNOON*

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The pond snail *Lymnaea stagnalis* acquires conditioned taste aversion (CTA) and maintains its memory for more than a month. Snails in our laboratory were cultured at 20 °C on a 12 : 12 light-dark cycle (light from 7 am to 7 pm). To examine the hours during which snails acquire CTA effectively, we trained some snails in the morning and others in the afternoon, and then compared their scores. CTA developed in both cases, but scores were significantly better in the morning than in the afternoon. To elucidate the cause of this difference in scores, we observed the voluntary activity of snails and found the circadian rhythm reflected in the snails' free-movement distances; distances at the circadian time 0–12 (daytime) were significantly longer than those at the circadian time 12–24 (nighttime). This rhythm was kept up for at least 3 days, even in constant darkness. In conclusion, *L. stagnalis* should be trained in the morning to acquire associative learning, possibly because of its greater propensity to roam about at that time as opposed to the afternoon.

Keywords: Associative learning – circadian rhythm – *Lymnaea*

INTRODUCTION

Not only can the pond snails *Lymnaea stagnalis* be classical-conditioned [1, 9, 10, 16, 25], but they are also amenable to operant conditioning [11, 20, 21]. In particular, they can exhibit a type of aversive classical conditioning, referred to as conditioned taste aversion (CTA) [14]. In naive control snails, application of sucrose (the conditioned stimulus, CS) always induces a feeding response, that is, a large number of bites, whereas KCl (the unconditioned stimulus, US) evokes a withdrawal

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response and suppresses feeding [14]. By contrast, conditioned snails show a weaker feeding response to sucrose for more than a month [14].

To elucidate the cellular and molecular mechanisms underlying CTA, we have so far employed several techniques against molecules to whole snails: electrophysiology [2, 23], optical imaging [17, 18], histology [5, 6, 7, 22], molecular cloning [28] as well as developmental approaches [27, 29, 30]. The most important findings are that (*a*) the cerebral giant cell plays a crucial role in CTA [15]; (*b*) the cascade involving cyclic AMP (cAMP), protein kinase A and cAMP responsive element binding protein (CREB) functions in the cerebral giant cell [24, 28]; and (*c*) nitric oxide generated from the B2 motoneuron disturbs the feeding rhythm [12, 13, 26]. On the other hand, the behavioral data for CTA arose another question whether there are hours during which *L. stagnalis* acquires CTA effectively. Needless to say, the training is always performed in the blind protocol in the daytime from 7 am to 7 pm, which corresponded to the lighting time in our culture room.

In the present study, we compared scores of CTA of *L. stagnalis* between morning and afternoon. Scores were significantly better in the morning session than in the afternoon session. Further, we examined the circadian rhythm of voluntary activity. Our present findings suggest that *L. stagnalis* can be effectively trained in the morning to acquire CTA, possibly because of its greater propensity to roam about at that time as opposed to the afternoon.

MATERIALS AND METHODS

CTA learning procedure

We used adult snails *L. stagnalis* of 20–30 mm shell length. They were reared locally and fed with lettuce, and maintained at 20 °C under a 12 : 12 light-dark cycle (light from 7 am to 7 pm). Snails were trained by a CTA-learning procedure that was modified from that previously described [14]. Briefly, training was performed in a 60 mm Petri dish (Fig. 1A). The CS and US were a 5 ml solution of 10 mM sucrose and a 5 ml solution of 10 mM KCl, respectively, that were poured into the Petri dish by a pipette for 15 s with a 15 s interstimulus interval (Fig. 1B). The pairing of the CS and the US was repeated 10 times with a 10 min intertrial interval. Before and after the training session, a 5 ml solution of 10 mM sucrose as the CS was applied to the lips and washed out by distilled water. Then, the feeding response was determined for 1 min (Fig. 1B, test). A backward-conditioned (US-CS) control group and a naive (distilled water only) control group were also employed (Fig. 1B). The procedure of training was performed in the blind protocol. The morning session was performed in the circadian time 0–12, that is, 7 am to 1 pm, and the afternoon session was in the circadian time 12–24, that is, 1 pm to 7 pm.

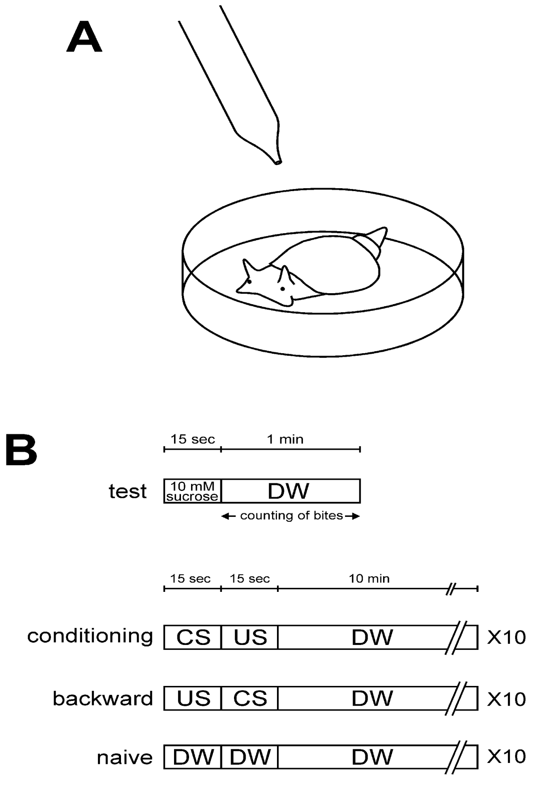

Fig. 1. CTA learning procedure. (A) Schematic diagram of the experimental apparatus. (B) Timing of CS and US in the training session

Voluntary activity

To examine whether there are any changes in activity other than the feeding response in snails in the daytime, we observed their voluntary activity. An individual snail was put into a dish (diameter = 21 cm) containing tap water, which was set in a black box (45 cm × 45 cm × 45 cm) equipped with a digital video camera (DCR-TRV9, SONY, Tokyo, Japan). The free moving for 1 h as the voluntary activity was recorded every 4 h. The sampling time was 5 s. The data were stored in a personal computer, and the free-movement distances were measured by ImageJ (NIH, Bethesda, MD, USA).

Statistical analyses

Data were expressed as mean ± SEM, and evaluated for statistical significance ($P < 0.05$) using one-factor ANOVA and Student's *t*-test.

RESULTS

CTA

In naive control snails, a 10 mM sucrose solution induced a large number of bites, and a 10 mM KCl solution evoked a withdrawal response. After sucrose stimulus was paired with the KCl stimulus in the CTA training session, the feeding response to sucrose was significantly reduced ($P < 0.001$ by one-factor ANOVA), compared to that observed when the backward-conditioned control procedure was employed, or when none of the stimulus were presented (naive control) (Fig. 2, the numbers of snails were 177, 150 and 158 for naive, backward and CTA, respectively).

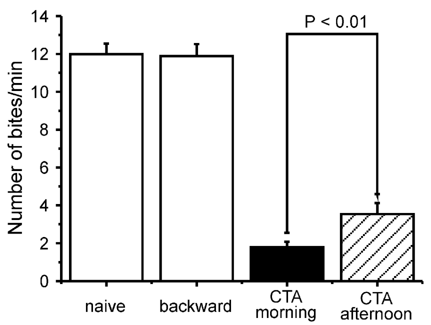

Fig. 2. Behavioral results of CTA

Interestingly, when we compared the scores of conditioned snails that were trained in the morning with those in the afternoon, we found that they were significantly better in the morning session than in the afternoon session ($P < 0.01$ by Student's *t*-test) (Fig. 2, the numbers of snails were 77 and 81 for the morning session of CTA and the afternoon session of CTA, respectively).

Circadian rhythm

Snails voluntarily moved in the dish at a speed of about 1–3 m/h all the day (Fig. 3). When we compared the free-movement distances at the circadian time 3 with those at the circadian time 15 (that is, 10 a.m. and 10 p.m., respectively), we found that the free-movement distances were significantly longer in the morning than in the night ($P < 0.05$ by Student t-*t*est) (Fig. 3, the numbers of snails examined here were at least 11). According to this rhythm, the voluntary activity in the morning was enhanced and that in the afternoon was suppressed (Fig. 3).

Even in constant darkness, the circadian rhythm was kept up for at least 3 days (Fig. 4, the numbers of snails examined here were at least 4). There was no signifi-

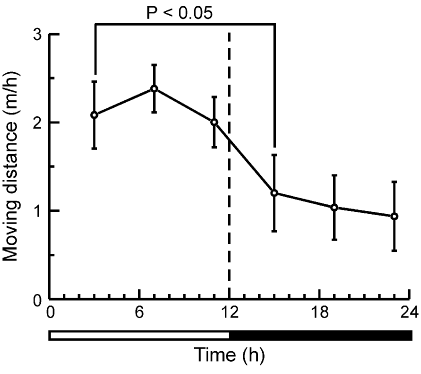

Fig. 3. Voluntary activity under the 12 : 12 LD condition

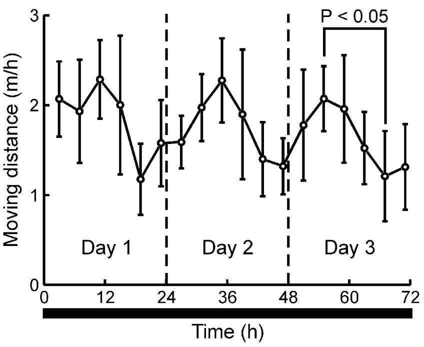

Fig. 4. Circadian rhythm under the constant dark condition

cant difference in the voluntary activity between the morning of Day 1 and that of Day 3, or between the night of Day 1 and that of Day 3.

DISCUSSION

We examined the hours during which snails acquire CTA effectively, and found that the scores are significantly better in the morning session than in the afternoon session (Fig. 2). Further, we found that the voluntary activity is changed according to the circadian rhythm, and that the activity is enhanced in the morning and suppressed in the afternoon (Fig. 3). We therefore conclude that snails should be trained in the morning to acquire associative learning. This fact may be due to snails' greater propensity to roam about in the morning, as opposed to the afternoon. Such a difference in the propensity between morning and afternoon originates from the circadian rhythm, which can be kept even in the constant darkness (Fig. 4).

Some lines of evidence have already been reported about the circadian and seasonal regulation of synapses and transcription factors involved in long-term memory in molluscs [3, 4, 8, 19]. For example, Eskin group has revealed that the mRNA level of CCAAT/enhancer binding protein (C/EBP, a transcription factor) in the eye of *Aplysia* are modulated by serotonin or light, and they concluded that C/EBP is a candidate gene for a circadian transcription factor to mediate circadian responses activated by second messengers [8]. We have already cloned cDNAs of CREB1, CREB2 and C/EBP in *L. stagnalis*, all of which are considered to play important roles in the long-term memory consolidation of CTA [28, also see the articles of Sadamoto et al. and Hatakeyama et al. in the same issue]. We should measure the changes in copy numbers of the mRNAs of these transcription factors during the daytime in future work.

We can easily construct a classical-conditioning paradigm with appetitive and aversive stimuli, such as CTA, in snails during the daytime. However, the scores are significantly better in the morning session than in the afternoon session. We therefore conclude that "the early snail acquires the learning".

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