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Effects of serotonin on the heartbeat of pond snails in a hunger state

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Serotonin (5-hydroxytryptamine: 5-HT) is a multimodal transmitter that controls both feeding response and heartbeat in snails. However, the effects of 5-HT on the hunger state are still unknown. We therefore examined the relation among the hunger state, the heartbeat rate and the 5-HT action in food-starved snails. We found that the hunger state was significantly distinguished by the heartbeat rate in snails. The heartbeat rate was high in the food-satiated snails, whereas it was low in the food-starved snails. An increase in 5-HT concentration in the body boosted the heartbeat rate in the food-starved snails, but did not affect the rate in the food-satiated snails. These results suggest that 5-HT application may mimic the change from a starvation to a satiation state normally achieved by direct ingestion of food.

Key words: food satiation, food starvation, heartbeat, *Lymnaea*, serotonin

The pond snail *Lymnaea stagnalis* is able to learn taste aversion and to consolidate it into long-term memory. This is referred to as conditioned taste aversion (CTA) [1–3]. Previous studies demonstrated that a modest food starvation period (i.e., a 1-day period) before training produced better and more consistent results [4–6]. However, prolonged food starvation for 5 days blocked taste aversion learning and its consolidation into long-term memory [4–6]. This difference may have been due to a difference in the motivation for learning while the snails struggle to devise a survival strategy [7].

During the observation of the severely food-starved snails (i.e., 5-day food starvation), we happened to observe that some of the snails showed slow heartbeats. This phenomenon was not thought to be due to the poor health condition (i.e., indisposition) of snails, because learning and memory formation for CTA indeed occurred in these snails, when they were injected with insulin [6]. On the other hand, several previous experiments showed that serotonin (5-hydroxytryptamine: 5-HT) is a key transmitter to control the feeding response and hunger state in various animals [8–12] including molluscs [13–19]. Further, the heartbeat rate can also be manipulated by application of 5-HT, its precursor and its antagonist [20–25]. We thus hypothesized that an increase in 5-HT concentration in the body boosts the heartbeat rate in food-starved snails.

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Materials and Methods

Snails

Specimens of *Lymnaea stagnalis* (L.) 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°C and fed *ad libitum* on turnip leaves (*Brassica rapa* var. *peruviridis*; Komatsuna [in Japanese]) and goldfish food (TetraFin Goldfish Flakes; Tetra Werke, Melle, Germany) every other day. *Lymnaea* exhibit good growth and reproduction under these conditions.

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Effect of serotonin on heartbeat

We prepared 6 groups of 10 snails each. Snails were individually placed in a plastic cup of 40 mm diameter and 100 mm height that contained pond water to a depth of 30 mm. 5-HT was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Group 1: This group had *ad libitum* access to food for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min for each snail in this group. The beating heart in *Lymnaea* was visible through the shell to the naked eye in all the experiments [26]. Then, the snails were again given *ad libitum* access to food in the pond water. During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the pond water, and again at the second test 24 h after immersion in the pond water. The snails were then given another 24 h of *ad libitum* access to food in the pond water, and the third test was performed by counting the number of heartbeats per min.

Group 2: This group had *ad libitum* access to food for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min for each snail in this group. Then, this group was again given *ad libitum* access to food in a 500 μM 5-HT solution. We mimicked this concentration in the studies using *Aplysia* [15, 16, 19]. During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the 5-HT solution, and again at the second test 24 h after immersion in the 5-HT solution. After the second test, the snails were washed completely with pond water and then given another 24 h of *ad libitum* access to food in the pond water, followed by a third test with counting of the number of heartbeats per min.

Group 3: This group had *ad libitum* access to food for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min for each snail in this group. The snails were then placed in pond water without food (food-starvation condition). During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the pond water, and again at the second test 24 h after immersion in the pond water. The snails were then food-starved for an additional 24 h in the pond water, and then the third test was performed by counting the number of heartbeats per min.

Group 4: This group was food-starved for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min. Then, this group was given *ad libitum* access to food in the pond water. During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the pond water, and again at the second test 24 h after immersion in the pond water. The snails were then given an additional 24 h of *ad libitum* access to food in the pond water, and then the third test was performed by counting the number of heartbeats per min.

Group 5: This group was food-starved for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min. Then, this group was immersed in a 500 μM 5-HT solution and subjected again to food starvation. During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the 5-HT solution, and again at the second test 24 h after immersion in the 5-HT solution. After the second test, the snails were washed completely with pond water, then food-starved for an additional 24 h in the pond water, followed by a third test with counting the number of heartbeats per min.

Group 6: This group was food-starved for 5 days before the pretest. At the pretest, the number of heartbeats was counted for 1 min. The snails were then again food-starved in the pond water. During this period, the number of heartbeats per min was counted at the first test 1.5 h after immersion in the pond water, and again at the second test 24 h after immersion in the pond water. Once again, the snails were food-starved for 24 h in the pond water, and then the third test was performed by counting the number of heartbeats per min.

Statistics

The data are expressed as the means ± SEM. Significant differences at $P<0.05$ were examined by Mann-Whitney U-test for two independent samples and by two-way repeated ANOVA and post hoc Tukey's test for three independent samples.

Results and Discussion

The difference in the heartbeat rate between the food-satiated snails and the food-starved snails was significant (Fig. 1). The results showed that the heartbeat rate in the food-satiated snails was higher ($P<0.01$) than that in the food-starved snails (i.e., the snails subjected to 5-day starva-

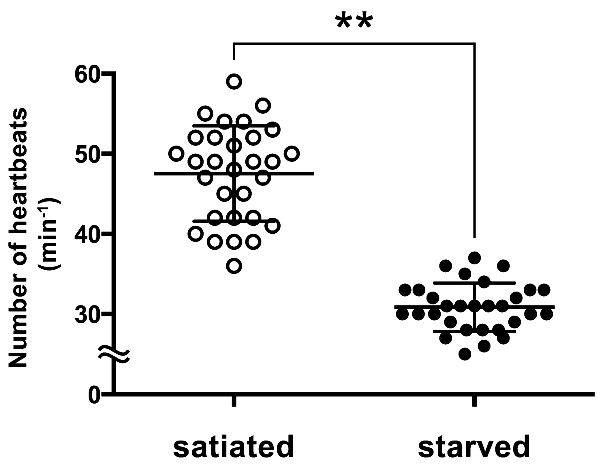

Figure 1 Difference in the number of heartbeats between food-satiated and food-starved snails. The food-starved snails did not get access to food for 5 days. The data were obtained as the results of the pretest in Figures 2 and 3. The horizontal bars show the means ± SEM. The number of snails was 30 in each group. The difference was significant (**$P<0.01$).

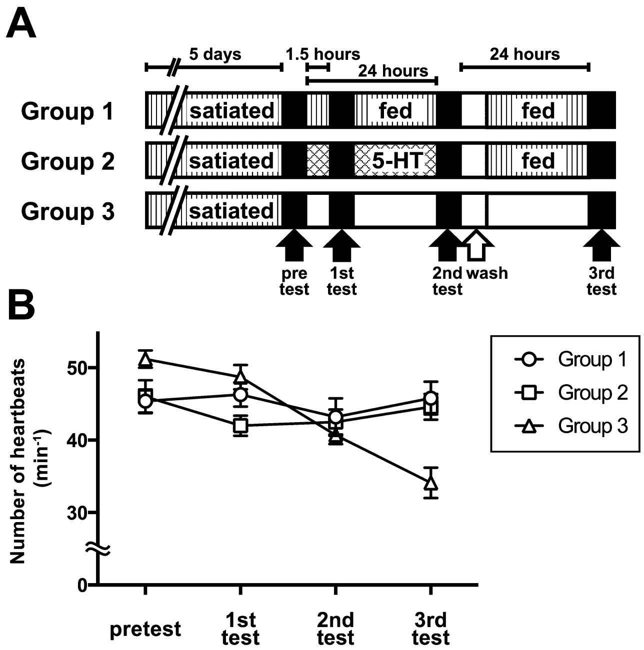

Figure 2 Effects of 5-HT and food starvation on the heartbeats of food-satiated snails. (A) Experimental protocols for food-satiated snails. We prepared 3 groups, Group 1, Group 2 and Group 3, as indicated in the text. (B) The food deprivation significantly decreased the number of heartbeats at the third test ($P<0.01$, Group 3 vs. (Group 1 or Group 2)), whereas the application of 5-HT did not affect it (no significance was found for Group 2 vs. Group 1).

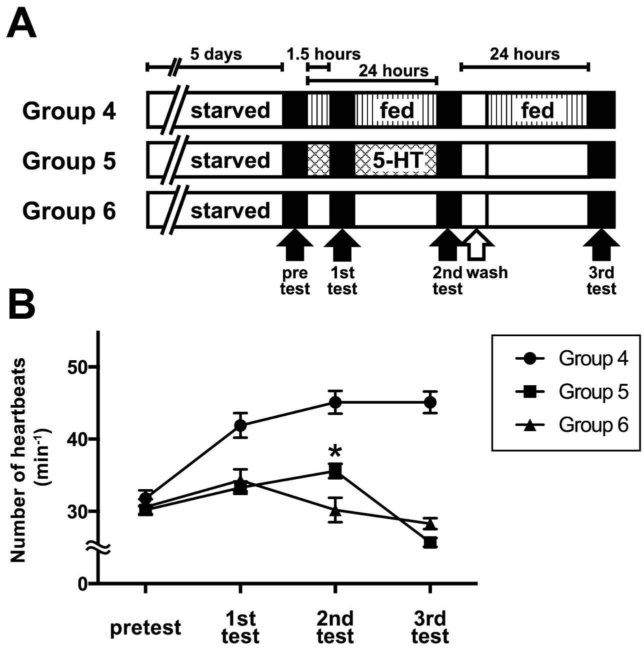

Figure 3 Effects of 5-HT and food satiation on the heartbeats of food-starved snails. (A) Experimental protocols for food-starved snails. We prepared 3 groups, Group 4, Group 5 and Group 6, as indicated in the text. (B) The food satiation significantly increased the number of heartbeats at the third test ($P<0.01$, Group 4 vs. (Group 5 or Group 6)). The data at the second test were especially noteworthy. The application of 5-HT also significantly increased the heartbeat rate (*$P<0.05$, Group 5 vs. Group 6), but there still remained a significant difference between Group 4 and Group 5 ($P<0.01$). That is, 5-HT partially boosted the heartbeats in the food-deprived snails.

tion), resulting in a clear distinction between the two cohorts. As described above, this difference was not due to the poor health condition in the food-starved snails.

We examined the effects of 5-HT and food starvation on the heartbeat rate in the food-satiated snails (Fig. 2). Here we prepared 3 groups: Group 1 was a control group; Group 2 contained the snails for examination of the effect of 5-HT; and Group 3 contained the snails for examination of the effect of food starvation. At the first test that was performed 1.5 h after the respective treatment, there was no significant difference between Group 1 and Group 3, but there was a significant difference between Group 2 and Group 3 ($P<0.05$). This effect did not seem to have been brought about by 5-HT, because this was observed in the slightly but significantly decreasing process according to the immersion time (i.e., a new environment). At the second test that was performed at 24 h after the respective treatment, the heartbeat rates in all the groups decreased slightly in comparison with those at the pretest (for example, $P<0.01$ between the pretest and the second test in Group 3). Because the control Group 1 also showed this decrease, this change was thought to be within the degree of dispersion of the data.

At the third test in Figure 2, Group 3, which was food-starved for the previous 24 h, showed a significant decrease in the number of heartbeats compared with the numbers in Group 1 and Group 2 ($P<0.01$). These results showed again that food starvation decreased the heartbeat rate. We should note that Group 2, which was treated with 5-HT, did not show any change in the heartbeat rate. This fact is important and will be discussed later.

We next examined the effects of 5-HT and food satiation on the heartbeat rate in the food-starved snails (Fig. 3). We prepared 3 other groups: Group 4 snails were used to examine the effect of food satiation; Group 5 snails were used for examination of the effect of 5-HT; and Group 6 was a control group. At the first test, which was performed at 1.5 h after the respective treatment, there was a significant difference between Group 4 and the other groups (Group 5 and Group 6) ($P<0.01$). At the second test, which was performed at 24 h after the respective treatment, the application of 5-HT significantly increased the heartbeat rate ($P<0.05$) in comparison with that in the control group, Group 6. Because there was still a significant difference ($P<0.01$) between the 5-HT-treated snails (Group 5) and the fed snails (Group 4), we consider that 5-HT "partially" boosts the heartbeat rate in the food-starved snails.

At the third test in Figure 3, Group 4 snails, which had been food-satiated for the previous 48 h, showed a significantly higher number of heartbeats in comparison with those in Group 5 and Group 6 ($P<0.01$). These results again showed that food satiation increased the heartbeat rate. Because Group 5 demonstrated a decrease of the heartbeat rate, the application of 5-HT was thought to have a short-term effect on the snails.

The present findings clarified the following 3 points.

(1) The heartbeat rate was higher in the food-satiated snails than in the food-starved snails.
(2) The heartbeat rate was changed according to the satiation/starvation state of snails.
(3) An increase in 5-HT concentration in the body boosted the increase in the heartbeat rate in food-starved snails. But this effect lasted only for the short term.

The results that 5-HT boosted the heartbeat rate in the food-starved snails do not rule out the possibility of a direct effect of 5-HT on the heart. However, when we applied 5-HT to the food-satiated snails, we did not obtain any increase in the heartbeat rate at the first, second or third tests (see Group 2 in Fig. 2) even though the heartbeat rate may not have reached its maximum value (the maximum rate was estimated as ca. 60 beats/min; Fig. 1). Therefore, the effect of 5-HT on the heartbeat rate in the food-starved snails may first change from the starvation state to the satiation state in the internal state of snails and then increase the heartbeat rate.

The action of insulin in the central nervous system of *Lymnaea* is involved in the feeding response in the starvation/satiation state [5, 6, 27–30]. Although no direct evidence has been shown for the interaction between 5-HT and insulin in snails, some interactions between these in the hypothalamus of mammals have been demonstrated [31]. For example in the median hypothalamus, there are the bidirectional effects of 5-HT and insulin. This interaction has been thought to be a link in a larger cascade of events in the complex regulatory loop between hypothalamic neuromodulators and nutritional behavior.

Finally, we should consider the "skipped heartbeat" phenomenon, which is known to occur during CTA in *Lymnaea* [26]. In taste aversion training for snails, the conditioned stimulus (CS; e.g., sucrose) that elicits a feeding response is paired with the unconditioned stimulus (US; e.g., electric stimulus or KCl) that elicits the whole-body withdrawal response and inhibits feeding. After acquiring CTA, the CS no longer elicits feeding. We hypothesized that one reason for this result is that after taste aversion training the CS elicits a "fear response". Consistent with this hypothesis, we predicted the CS would cause (1) the heart to skip a beat and (2) a significant change in the heartbeat rate. We found that in snails exhibiting CTA and long-term memory the CS significantly increased the probability of a "skipped heartbeat", but did not significantly change the heartbeat rate. The probability of a skipped heartbeat was unaltered in control snails given backward conditioning (US followed by CS) or in snails that did not acquire CTA. These results suggested that as a consequence of acquiring CTA, the CS evokes "conditioned fear" in the CTA snails, as evidenced by a change in the nervous system control of cardiac activity.

In conclusion, to our knowledge, this is the first report across phyla to discuss the relation among the hunger state, the heartbeat rate and the action of 5-HT.

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Conflict of Interest

All the authors declare that they have no conflict of interest.

Author Contributions

E.I. directed the entire project. M.Y. and E.I. performed the experiments. M.Y., T.W., D.H. and E.I. analyzed the data. M.Y., T.W., D.H. and E.I. co-wrote the manuscript.

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