Brief Communication

Depletion of serotonin selectively impairs short-term memory without affecting long-term memory in odor learning in the terrestrial slug *Limax valentianus*

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The terrestrial slug *Limax* is able to acquire short-term and long-term memories during aversive odor-taste associative learning. We investigated the effect of the selective serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) on memory. Behavioral studies indicated that 5,7-DHT impaired short-term memory but not long-term memory. HPLC (high-performance liquid chromatography) analysis revealed that 5,7-DHT significantly reduced serotonin content in the central nervous system. The present study suggests that acquisition, retention, and/or retrieval of short-term memory involves serotonin, and neither acquisition nor retrieval of long-term memory requires serotonin at a level as high as that required for short-term memory.

The land slug *Limax* has a highly developed olfactory system and exhibits aversive odor-taste associative learning (Sahley et al. 1981). It avoids the odor of innately attractive food (e.g., carrot or cucumber), the conditioned stimulus (CS), following conditioning by a paired presentation of the CS and the bitter-taste of quinidine, which is a noxious unconditioned stimulus (US). This behavioral change persists for at least two weeks even after only one conditioning trial (Matsuo et al. 2002). Extensive behavioral analyses have been done, and the properties of the memory have been characterized (Sahley et al. 1981; Yamada et al. 1992). The central networks involved in odor processing and memory have also been extensively studied by physiological methods and are well documented (Gelperin and Tank 1990; Kimura et al. 1998a,b,c; Watanabe et al. 1998, 1999, 2003; Inoue et al. 2004). However, the molecular mechanisms for this learning are only partly understood. Elucidation of the neurotransmitters and neuromodulators, especially those involved in learning, will be a key step toward understanding the mechanisms of odor learning.

Based on previous findings, serotonin is likely to play an important role in learning. First, the procerebral (PC) lobe of *Limax*, which is the putative site where learning is thought to occur (Kimura et al. 1998a), receives serotonergic projections (Osborne and Cottrell 1971; Inoue et al. 2004). Many serotonin-containing neurons are found in the central nervous system (CNS) of *Limax* (Shirahata et al. 2004). Second, biochemical and electrophysiological changes in the PC lobe are triggered by serotonin (Yamane and Gelperin 1987, 1989; Gelperin et al. 1993). Also, in *Aplysia* (another mollusk), serotonin is essential for gill withdrawal learning (Glanzman et al. 1989), although this is a simple reflex type of learning. However, there have been no behavioral data that directly indicate whether serotonin is involved in the odor learning of *Limax*. In the present study, we asked if serotonin is actually involved in aversive odor-taste associative learning.

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Memories are divided into short-term and long-term phases. Memory in *Limax* is not affected by protein synthesis inhibitors up to one day after conditioning, while memories that persist two or more days after conditioning are (Matsuo et al. 2002; Yasui et al. 2004). Therefore, memory until one day after conditioning should be categorized as short-term, while memory that endures after this time should be considered long-term. Since different phases of memory may involve different molecular mechanisms, we also may find differential dependence on serotonin of each memory phase. The next step will be to clarify the dependence of each memory phase on serotonin.

We conducted behavioral experiments using the selective serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) (Daly et al. 1973; Elekes et al. 1977; Baumgarten et al. 1982; Glover and Kramer 1982; Glanzman et al. 1989). 5,7-DHT produces a gradual and long-lasting reduction in the serotonin content of central neurons. In *Helisoma*, depletion of serotonin by 5,7-DHT persists for approximately one month and the normal level of serotonin is restored in two months. Depletion of serotonin occurred both in the axons and the somata, and a greater effect was observed in the former (Gadotti et al. 1986).

Terrestrial slugs (*Limax valentianus*) are maintained in our laboratory and animals weighing 0.5–1.0 g (8–16 wk after hatching) were used in the experiments (Matsuo et al. 2002). Aversive odor-taste conditioning was performed as follows: 1 mL of a mixture of equal volumes of carrot juice (prepared from carrot using a juicer, stored at −20°C) and saturated (~1% w/v) quinidine sulfate (Wako Pure Chemicals) solution was put on a glass plate in the shape of a half-circle arc with a 6-cm radius around a marked point on the glass plate. Since quinidine solution is odorless to this species, the odor of the mixture is that of carrot. Next, the slug was placed on the glass plate with its head on the marked point. After we confirmed that the slug had begun to crawl toward the mixture and its mantle had passed the marked point, we observed its behavior and confirmed that all the slugs touched the mixture. As each slug touched the mixture, it withdrew its head, and this was used to indicate that the slug had sensed the US. After the head of the slug touched the mixture, the slug was kept in contact with the mixture for 90 sec on the glass plate and then submerged and rinsed in water for 30 sec. Finally, it was

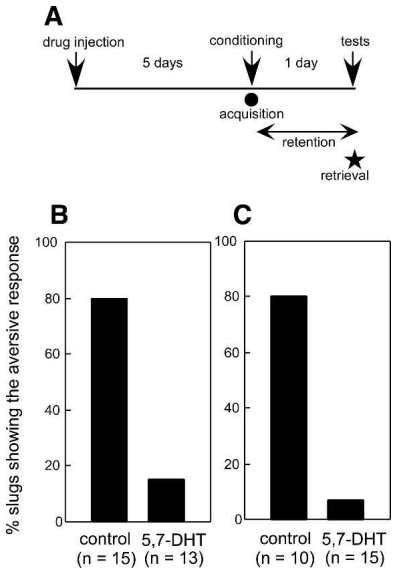

Figure 1. Effect of 5,7-DHT on short-term memory. (*A*) Time course of the experiments. Conditioning was carried out five days after injection of vehicle solution (control group) or 5,7-DHT solution (5,7-DHT group). One day after conditioning, CR, mobility, and odor sensitivity tests were performed. We assumed that acquisition of short-term memory occurs during conditioning (circle), the retrieval occurs at the CR test (star), and retention occurs between the acquisition and the retrieval (arrow). (*B*) Percentage of aversive response in carrot-conditioned slugs. The 5,7-DHT group showed a significantly lower percentage than the control group. $P = 0.0009$ by Fisher's exact probability test. (*C*) Percentages of aversive response in cucumber-conditioned slugs. The 5,7-DHT group showed a significantly lower percentage than the control group. $P = 0.0003$ by Fisher's exact probability test.

transferred to a plastic container and kept in an incubator at 20°C.

We injected a 10-mg/mL solution of 5,7-DHT creatinine sulfate salt (Sigma) (final dose ~1–2 mg/g b.w.) dissolved in vehicle solution into the body cavities of the slugs in the 5,7-DHT group. The vehicle solution contained 10 mg/mL sodium L-ascorbate (Wako Pure Chemicals) as an antioxidant for 5,7-DHT, dissolved in saline (Matsuo et al. 2002). We also injected the vehicle solution into the control subjects in the same way.

According to a previous study, 5,7-DHT is effective at 5 d after injection (Glanzman et al. 1989). We also confirmed this in the present study using high-performance liquid chromatography (HPLC) analysis, as described below. The experiments were designed so that the targeted step (conditioning or test) was carried out on the fifth day after the injection of 5,7-DHT.

To examine the effect of 5,7-DHT on short-term memory (Fig. 1A), conditioned response (CR) tests were performed 24 h after the conditioning. For the CR test, 1 mL of carrot juice was placed in an identical way to that used during conditioning. We then observed whether the head of the slug touched the juice. If the slug exhibited an aversive response (the head of the slug did not touch the juice within 180 sec) after its mantle passed the marked point, this was judged to be an aversive response. If it did not show an aversive response within 180 sec, this was recorded as the absence of a response. The slugs were then subjected to a mobility test to confirm their locomotor ability and to odor sensitivity tests to verify their odor sensing ability. The mobility test was performed in the same way as the CR tests, except that suspension of rat chow (the slugs' customary daily food, type MF, Oriental Yeast; suspended in water to 0.5 g/mL just before use) was used instead of carrot juice. For the odor sensitivity test, we used garlic homogenate (an aversive odor source for slugs, prepared from garlic and stored at −20°C). In each test, we determined whether an aversive response occurred (or not) within 180 sec. To examine the effects of 5,7-DHT on long-term memory (Fig. 2A), CR tests were performed 6 d after the conditioning in a similar way. To investigate the effect of 5,7-DHT on the retrieval of long-term memory (Fig. 2C), 5,7-DHT or the vehicle solution (controls) was injected one day after conditioning. Tests were performed 6 d after the conditioning. Injection of the drug, the conditioning, and the tests were performed in the same way as described above.

The decrease in serotonin content caused by the 5,7-DHT treatments in the central ganglia (the cerebral ganglia and the subesophageal ganglia) was quantified by HPLC. The central ganglia were isolated from each animal 5 d after injection of the 5,7-DHT or vehicle solution and transferred to a glass tissue grinder tube containing 700 µL of 0.1 M acetic acid. This tube was heated for 5 min at 100°C, after which the ganglia were homogenized. The homogenate then was transferred to a 1.5-mL tube. Other 300 µL of 0.1 M acetic acid were added to the grinding tube and the tube was vortexed to rinse any remaining homogenate from its walls. This acetic acid rinse was also transferred to the 1.5-mL tube. The homogenized ganglia were centrifuged for 30 min (1300 rpm at 4°C) and the supernatant was lyophilized. Each sample (containing the extract of the central ganglia from a single animal) was redissolved in 200 µL of 0.1 M acetic acid. The HPLC analysis was performed using a PU-2080 pump (Jasco) and a NANOSPACE SI-2 electrochemical detector (Shiseido). Serotonin was eluted with a CAPCELLPAK C18

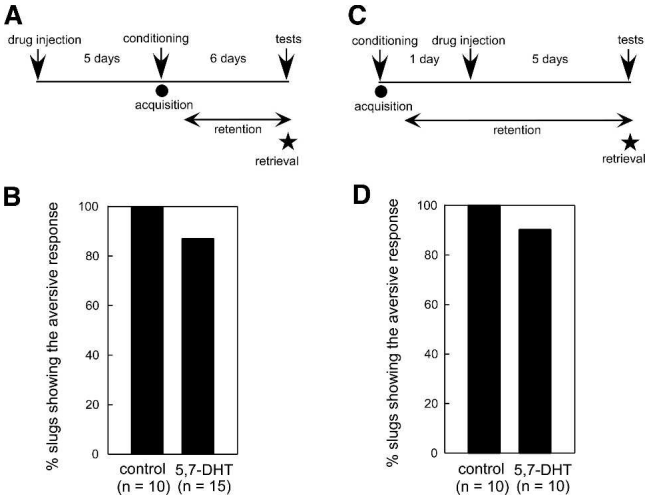

Figure 2. Effect of 5,7-DHT on long-term memory. Vehicle solution was injected into the slugs in the control group, while 5,7-DHT solution was injected into the 5,7-DHT animals. We assumed that acquisition of long-term memory occurs during a period from the onset of conditioning to 2 h after the end of conditioning (circle), according to Yasui et al. (2004), that retrieval occurs at the CR test (star), and that retention occurs between the acquisition and retrieval (arrow). (*A*,*B*) Effects of 5,7-DHT injected 5 d before the conditioning. Five days after injection, conditioning was performed, and six days after conditioning, CR, mobility, and odor sensitivity tests were performed. The time course of the experiments (*A*) and the results (*B*) are shown. The percentage of slugs that showed an aversive response was not significantly different between the drug- and vehicle-injected groups. $P > 0.05$ by Fisher's exact probability test. (*C*,*D*) Effects of 5,7-DHT injected one day after conditioning. One day after conditioning, drug injection was performed. Five days after drug injection, CR, mobility, and odor sensitivity tests were conducted. The time course of the experiments (*C*) and the results (*D*) are shown. The percentage of slugs that showed an aversive response was not significantly different between the drug- and vehicle-injected groups. $P > 0.05$ by Fisher's exact probability test.

UG-120 1.5 × 250 mm column (Shiseido). The mobile phase consisted of 50% acetonitrile with 25 mM formic acid and 10 mg/L of EDTA, adjusted to pH 3.0–3.5, and then vacuum-degassed and filtered. The flow rate through the column was 100 µL/min. The potential across the electrode of the detector was 0.7 V. We recorded the current output of the detector on chart paper. The serotonin content of the sample peaks was calculated by comparing them with HPLC peaks from external standards of serotonin run at the same time as the samples.

First, the effect of 5,7-DHT on short-term memory was examined using carrot juice as CS. In the control group, 80% of the slugs ($n = 15$) showed an aversive response (CR) to the carrot juice in the CR test performed one day after the conditioning, while none of them showed an aversive response to the rat chow in the mobility test, indicating that the control group was conditioned selectively to the CS. In contrast, only 15% of the slugs ($n = 13$) in the 5,7-DHT group exhibited an aversive response in the CR test, which was significantly different from the responses of the control group ($P = 0.0009$, Fisher's exact probability test, Fig. 1B). Moreover, all of them showed an aversive response in the odor sensitivity test, suggesting that the olfactory system was not damaged in the 5,7-DHT group. None of these animals gave an aversive response in the mobility test, and the times to reach the rat chow were not significantly different between the control and 5,7-DHT groups (20.7 ± 2.0 sec for the control group, 23.0 ± 3.0 sec for the 5,7-DHT group, $P > 0.05$, Student *t*-test). All the slugs in both the control and 5,7-DHT groups showed similar withdrawal response when their heads touched the mixture during conditioning, suggesting that the slug's ability to sense the US was approximately equal in the two groups. In the experiment using cucumber as CS, 80% of the slugs ($n = 10$) in the control group showed an aversive response to cucumber juice in the CR test. In contrast, only 7% of the slugs ($n = 15$) in the 5,7-DHT group exhibited an aversive response in the CR test, which was significantly different from the responses of the control group ($P = 0.0003$, Fisher's exact probability test, Fig. 1C).

The effect of 5,7-DHT on the acquisition of long-term memory (Fig. 2A) was as follows. As in the case of short-term memory, 100% of the slugs ($n = 10$) in the control group that were injected with vehicle solution 5 d before the conditioning gave an aversive response in the CR test performed 6 d after the conditioning, while none of them produced an aversive response in the mobility test, indicating that the control group was conditioned selectively to the CS. In contrast to the case of short-term memory, 87% of the slugs ($n = 15$) in the 5,7-DHT group that were injected with 5,7-DHT solution 5 d before the conditioning showed an aversive response in the CR test 6 d after the conditioning. This was not significantly different from the control group ($P > 0.05$, Fisher's exact probability test, Fig. 2B). None of them produced an aversive response in the mobility test.

The effects of 5,7-DHT on the retrieval of long-term memory (Fig. 2C) were examined with the slugs that were injected with 5,7-DHT one day after the conditioning. In the CR test performed 6 d after the conditioning, 100% of the slugs ($n = 10$) in the control group that were injected with vehicle solution gave an aversive response, and 90% of the slugs ($n = 10$) in the 5,7-DHT group showed an aversive response in the CR test. This was not significantly different from the control group ($P > 0.05$, Fisher's exact probability test, Fig. 2D). None of the animals produced an aversive response in the mobility test.

The HPLC experiments indicated that the mean ± SEM serotonin content of the central ganglia from the control group ($n = 4$) was 132 ± 28 pmol, while that from the 5,7-DHT group ($n = 4$) was 60 ± 10 pmol. This reduction in serotonin content (45% of the control group) caused by injection of 5,7-DHT was statistically significant ($P = 0.025$, one-tailed unpaired Student *t*-test, Fig. 3).

The present study has clearly demonstrated that the injection of 5,7-DHT impairs either the acquisition, retention, or retrieval of short-term memory during aversive odor-taste associative learning in the slug. Unlike short-term memory, 5,7-DHT treatment does not impair the acquisition or retrieval of long-term memory. These results suggest either that serotonin is not involved in the acquisition or retrieval of long-term memory, or that a lower amount (less than 45%) of serotonin remaining after the treatment with 5,7-DHT is enough for long-term memories. Because responses to rat chow in the mobility test and that to garlic in the odor sensitivity test were similar between the control and 5,7-DHT groups, and the withdrawal response appeared to be similar, 5,7-DHT is unlikely to have significant nonlearning dependent effects.

The present results also suggest that short-term and long-term memories are processed independently, and that the latter can be established in the absence of the former. In *Aplysia*, two independent parallel processing pathways for short-term and long-term memories have been demonstrated. Although both short-term and long-term memories depend on serotonin, the serotonin antagonist cyproheptadine does not block the induction of long-term memory, but it does block the induction of short-term memory (Emptage and Carew 1993). In the marine mollusk *Hermissenda* serotonin-induced short-term enhancement of the generator potential in identified photoreceptors can be blocked without affecting long-term enhancement in the same cells (Crow and Forrester 1993). Although these examples are similar to the present results, the underlying neural networks are quite different. Odor learning in *Limax* may require a much more complex neural system, the PC lobe, which contains about 10^5 neurons and exhibits network dynamics common to higher cognitive centers (Gelperin and Tank 1990).

Another example of the parallel processing of short-term and long-term memories is found in *Drosophila*, whose memory one day after conditioning is disrupted by mutation of the *radish* gene, which encodes phospholipase-A2 (Chiang et al. 2004); 2–7 d after conditioning, memory is not disrupted by the mutation (Tully et al. 1994). In this case, the upstream transmitters are not known. Distinct pharmacological effects on short-term and long-term memories have also been reported in the one-trial step-down inhibitory avoidance task in rat (Izquierdo et al. 2002). In humans, some clinical conditions can result in impaired short-term memory while normal long-term memory re-

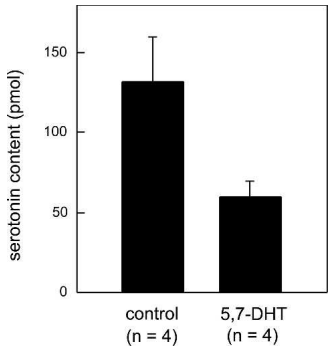

Figure 3. HPLC analysis of the serotonin content of the central ganglia from control and 5,7-DHT-treated slugs. The graph shows the mean value of the serotonin content for the two groups; error bars represent the SEM. The difference in the serotonin content between the ganglia from the controls and the 5,7-DHT-treated slugs was statistically significant ($P = 0.025$, one-tailed unpaired Student *t*-test).

mains intact (Baddeley and Warrington 1970; Shallice and Warrington 1970).

Our results indicate that aversive odor learning in *Limax* requires serotonin, and short-term and long-term memories are distinguished by their differential dependence on serotonin. Because the CNS of *Limax* is easily studied with electrophysiologial techniques in an isolated brain preparation (Inoue et al. 2004), *Limax* appears to be an ideal model animal for studying the phases of memory during odor learning.

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