

Brief Communication

Nitric oxide is involved in appetitive but not aversive olfactory learning in the land mollusk *Limax valentianus*

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The land slug *Limax* performs both aversive and appetitive olfactory learning, and we investigated neurotransmitters involved in each type of learning. Slugs were conditioned by presenting a vegetable juice (appetitive conditioning) or a mixture of vegetable juice and quinidine (aversive conditioning), and the latency to reach the juice became shorter (appetitive conditioning) or longer (aversive conditioning) after conditioning. L-NAME injected either before conditioning or testing blocked the reduction in latency in appetitive conditioning but had no significant effects in aversive conditioning. 5,7-dihydroxytryptamine had no significant effects in appetitive conditioning. These results suggest different mechanisms for appetitive and aversive learning.

The land mollusk *Limax* has a highly developed olfactory system and performs olfactory learning. Both aversive (Gelperin 1975; Sahley et al. 1981) and appetitive (Sahley et al. 1990) olfactory learning have been reported, and therefore, it is possible to compare these two forms of learning in *Limax*. Aversive learning occurs when an odor is associated with an aversive chemical or electrical shock, and appetitive learning occurs when an odor is associated with food. So far, aversive learning has been more extensively studied than appetitive learning. Our previous study showed using the serotonergic neurotoxin 5,7-dihydroxytryptamine (DHT), which reduces the serotonin content in the CNS to less than half, that serotonin is essential for aversive learning (Shirahata et al. 2006). However, it is not known whether appetitive learning also depends on serotonin, or whether it requires other neurotransmitters.

Nitric oxide (NO) synthase is abundant in the procerebrum (PC) (Fujie et al. 2002, 2005), which is a region of the cerebral ganglion essential for olfactory learning (Kasai et al. 2006). NO applied extrinsically modulates the rhythmic activity of the local field potential (LFP) of the PC (Gelperin 1994; Gelperin et al. 2000). These observations suggest significance of NO in olfactory processing in *Limax*. In *Helix pomatia*, a related land mollusk, appetitive learning is blocked by N^ω-nitro-L-arginine methyl ester (L-NAME), a blocker of NO synthase. In the present work, we examine using L-NAME whether NO is involved in appetitive and aversive learning in *Limax*. We examine dependence of the acquisition and retrieval stages on NO separately. We also examine dependence of appetitive learning on serotonin using DHT.

Limax valentianus 2–4 mo old from the laboratory colony were used. The slugs were freely fed with a mixture of rat chow (Oriental Yeast), wheat starch (Wako Pure Chemical), and mixed vitamins (Oriental Yeast), until 5 d before the experiment. The slugs were kept under the light/dark cycle of 10 h/14 h, and conditioning and testing were done within 4 h from the onset of the dark period. Five days before conditioning, the slugs were moved to individual boxes floored with moistened filter paper and starved. The training and testing were made on a glass plate under dim light at room temperature (22°C). All the behavioral

tests were done by an experimenter who did not know the previous treatment of the animal.

For appetitive conditioning, the slugs were conditioned by feeding a vegetable juice. One milliliter of diluted carrot or cucumber juice (freshly prepared juice diluted with the same volume of water, stocked for up to ~1 wk at 4°C) was placed in a half-circle arc of 6-cm radius, and the slug was gently placed at the center, with its head oriented to the juice. The time for the slug to reach the juice was recorded (preconditioning latency). When the slug touched the juice, it was kept in contact for 30 sec and allowed to ingest the juice, and it was then moved back in the box. The slugs that did not touch the juice within 150 sec were excluded from the experiment. In the testing session on the next day, the time to reach the juice was recorded again (post-conditioning latency) (Fig. 1A). All the slugs reached the juice within 150 sec in the testing session.

When the conditioned slugs were tested with the same stimulus as in the conditioning, the post-conditioning latency was significantly shorter than the preconditioning latency (Fig. 1B). When the conditioned slugs were tested with the stimulus that had not been used for conditioning, the post-conditioning latency was not significantly different from the preconditioning latency (Fig. 1C). This indicates that after appetitive conditioning, the slug moves more rapidly to the conditioned odor as a result of learning, and suggests that this learning is specific to the conditioned odor.

Next we examined effects of L-NAME on appetitive learning. L-NAME (Sigma-Aldrich) was dissolved in saline at 5 mg/mL (~18 mM) and injected into the body cavity at 100 µL/g body weight (final dose 0.5 mg/g body weight) 1 h before conditioning or testing. The same volume of saline was used for control. The saline solution contained (in mM) 70 NaCl, 2 KCl, 4.9 CaCl₂, 4.7 MgCl₂, 5 glucose, and 5 HEPES (pH 7.6). This concentration of L-NAME is identical with that used in a previous study (Sakura et al. 2004) and is higher than that used in behavioral study in *H. pomatia* (0.075 mg/g body weight) (Teyke 1996), but it is lower than that previously shown to block the LFP oscillation in vitro (20 mM) (Gelperin 1994), if L-NAME is assumed to be uniformly distributed in the body.

Injection of L-NAME 1 h before appetitive conditioning blocked the reduction in the post-conditioning latency, while injection of saline did not block the reduction (Fig. 2A). The preconditioning latencies were not significantly different be-

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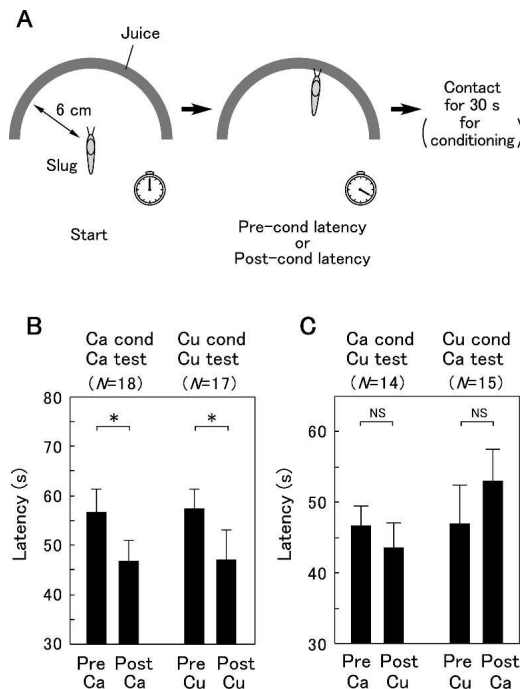


Figure 1. Appetitive odor conditioning. (A) Methods for conditioning and testing. For both conditioning and testing, vegetable juice was placed in a half-circle arc of 6-cm diameter, and the slug was placed at the center. The time for the slug to reach the juice was measured and recorded as preconditioning or post-conditioning latency. For conditioning, the slug was then kept in contact with the juice for 30 sec. (B) Reduction of post-conditioning latency after appetitive conditioning. Carrot (Ca)-conditioned (left) and cucumber (Cu)-conditioning slugs (right) were tested with the same conditioned odor. Error bars, SEM. The numbers of data are indicated above the graph. For both stimuli, the post-conditioning latency was significantly shorter than the preconditioning latency. Wilcoxon's signed ranks test, $*P < 0.05$. (C) Specificity of appetitive learning. Carrot-conditioned slugs were tested with cucumber (left), and cucumber-conditioned slugs tested with carrot (right). In both cases, the post-conditioning latency was not significantly different from the preconditioning latency. Wilcoxon's signed ranks test, NS indicates not significant ($P > 0.05$).

tween the L-NAME and control groups. Injection of L-NAME 1 h before testing also blocked the reduction in the latency (Fig. 2B). This indicates that L-NAME affects both acquisition and retrieval of memory in appetitive learning.

For aversive conditioning, the slugs were conditioned by presenting 1 mL of a mixture of carrot juice and quinidine (carrot juice mixed with the same volume of 1% solution of quinidine sulfate [Wako Pure Chemical] in water). When the slug touched the mixture, it was kept in contact for 30 sec and then rinsed in water for 1 min and moved back in the box. The slugs that did not touch the juice were excluded from the experiment. In the testing session on the next day, the preconditioning and post-conditioning latencies were measured in the same way as in appetitive conditioning. The post-conditioning latency for the slugs that did not touch the juice within 180 sec was defined as 180 sec.

In aversively conditioned slugs, the post-conditioning latency was longer than the preconditioning latency. Injection of L-NAME before conditioning did not have significant effects on the increase in the post-conditioning latency (Fig. 2C). Also, injection of L-NAME before testing did not have significant effects on the increase in the post-conditioning latency (Fig. 2D). These results indicate that L-NAME has no significant effect on either acquisition or retrieval of aversive learning.

We next examined effects of DHT on appetitive learning. DHT (Sigma) was dissolved in saline at 10 mg/mL together with ascorbic acid as antioxidant (10 mg/mL) and injected into the body cavity at 200 μ L/g body weight (final dose 2 mg/g body weight) 5 d before conditioning. The same volume of saline containing ascorbic acid was used for control. DHT gradually reduces the serotonin content in the CNS to ~40% at 5 d after injection (Shirahata et al. 2006). Because of the slow time course of DHT effect, DHT may affect both conditioning and testing.

The slugs injected with DHT 5 d before appetitive conditioning showed a significant reduction in the post-conditioning latency (Fig. 2E). The preconditioning latencies were not significantly different between the control and DHT groups. These results suggest that appetitive learning is possible in DHT-injected slugs.

The amount of L-NAME remaining in the CNS after injection was evaluated by recording modulation of the LFP oscillation in the PC, which is presumably mediated by NO. Stimulation of the superior tentacle nerve evoked an increase in the LFP frequency (Fig. 3A), and this was blocked by L-NAME. Although it is not yet clear whether the modulation of the LFP is related to odor processing or learning, we utilized this phenomenon merely to monitor effects of L-NAME in the CNS. After the injection of L-NAME, we dissected out the brain at various times and recorded the LFP frequency changes induced by stimulation of the superior tentacle nerve, within 20 min from dissection. The LFP was recorded from the posterior surface of the PC with a glass electrode filled with saline, which was connected to an amplifier (MEZ-2100, Nihon-Kohden) with a band pass filter of 0.5–30 Hz. Electrical stimulation was applied to the superior tentacle nerve from a glass suction electrode connected to an isolator (SS-403J, Nihon Kohden). The stimulus was a single pulse of 0.5 V, 1-msec duration. The timing of the stimulus was adjusted at the center of the interval between LFP peaks. The details of the protocols for dissection and LFP recording are explained elsewhere (Watanabe et al. 2003). The normalized frequency change was calculated using the average of the four LFP intervals before the stimulus, T_{pre} , and the interval between the first LFP peak that occurred at least 0.5 sec after the stimulus and the next peak, T_{post} , as $(T_{\text{pre}}/T_{\text{post}} - 1) \times 100$.

In the preparations from untreated slugs, stimulation of the superior tentacle nerve transiently increased the frequency of the LFP oscillation of the PC (Fig. 3A). In the preparations from the slugs injected with L-NAME and dissected 1–5 h later, the frequency increase was significantly smaller than in those from saline-injected controls. In contrast, the preparations made 23–25 h after injection of L-NAME showed similar levels of frequency increase as the controls (Fig. 3B). This indicates that CNS effects of L-NAME are absent at 24 h after injection, and suggests that L-NAME injected before conditioning does not affect the testing on the next day.

In the present work, we showed that L-NAME impairs both acquisition and retrieval of memory in appetitive learning but has no significant effect on aversive learning. In a previous report, L-NAME injected before testing impaired discrimination between aversively conditioned odors, but avoidance behavior to the conditioned odor was intact (Sakura et al. 2004). Also, motor responses recorded in an *in vitro* preparation from aversively conditioned slugs showed impaired odor selectivity in the presence of L-NAME, but the conditioned motor response itself was intact (Teyke and Gelperin 1999). Whether the effects of L-NAME on appetitive learning and discrimination have a common neural basis is an interesting question, which might be solved by future studies. The present results are partially consistent with a previous report in *H. pomatia*, in which L-NAME blocked the acquisition of food attraction learning (Teyke 1996). In *H. pomatia*, however, retrieval of memory was not blocked by L-NAME. It

should be noted that the dose of L-NAME used in the present study was higher, and one possible explanation for the discrepancy is that the acquisition and retrieval processes have different sensitivity to L-NAME. Differences in the species and the learning tasks could also account for the different results.

The electrophysiological experiment suggested that NO is released in the PC following stimulation of the odor input pathway (Fig. 3). Although NO synthase is also present in other regions of the nervous system (Fujie et al. 2002), NO released in the PC is a strong candidate for a mechanism involved in olfactory learning. NO is abundant in olfactory centers of both invertebrates and vertebrates, and in some animals, NO is involved in olfactory learning. For example, NO is released in the antennal lobe of insects, the first order olfactory center (Collmann et al. 2004) and modulates neural activity (Wilson et al. 2007). In the honeybee, NO is involved in habituation (Muller and Hildebrandt 2002) and long-term memory in associative olfactory learning (Muller 1996). NO also plays an important role in olfactory learning in infant rats (Samama and Boehm 1999) and sheep (Kendrick et al. 1997). Besides olfactory learning, NO is involved in various types of learning, including appetitive learning for chemical stimuli in *Lymnaea* (Kemenes et al. 2002) and tactile (Robertson et al. 1994) and visual (Robertson et al. 1996) learning

in the octopus. In *Aplysia*, NO is involved in memory on food (Katzoff et al. 2002). NO is also involved in other types of learning in vertebrates (Li et al. 1995; Kendrick et al. 1997; Qiang et al. 1997; Samama and Boehm 1999) and is known as essential for long-term changes in synaptic transmission (Schuman and Madison 1991; Daniel et al. 1993).

The effects of L-NAME in appetitive learning revealed in the present study are not due to its effect on locomotion, because the preconditioning latency was not different between saline and L-NAME groups (Fig. 2A,C). Also, since the animals injected with L-NAME avoided odors in aversive learning, the ability of the slug to sense odors is unlikely to be impaired.

Appetitive learning still occurred in animals injected with DHT. This suggests that, at least with the level of serotonin remaining after DHT administration, neither acquisition nor retrieval of appetitive memory is impaired. In contrast, DHT blocked the acquisition of aversive learning (Shirahata et al. 2006). Although DHT reduced the serotonin content in the central ganglia only partially, DHT may reduce serotonin in the synaptic regions more selectively and may severely impair serotonergic transmission (Gadotti et al. 1986). Therefore, appetitive learning is suggested to be either independent of serotonin or dependent on serotonin to a much lower extent than aversive learning. A previous report also showed absence of DHT effect on appetitive learning (Teyke 1996).

Differential contribution of neurotransmitter systems in appetitive and aversive learning has been known in insects; octopamine is involved in appetitive learning, whereas dopamine is involved in aversive learning (Farooqui et al. 2003; Schwaerzel et al. 2003; Keene and Waddell 2005; Unoki et al. 2005; Kim et al. 2007). In some forms of learning, mechanisms of acquisition and retrieval of memory are independent. In aversive conditioning in

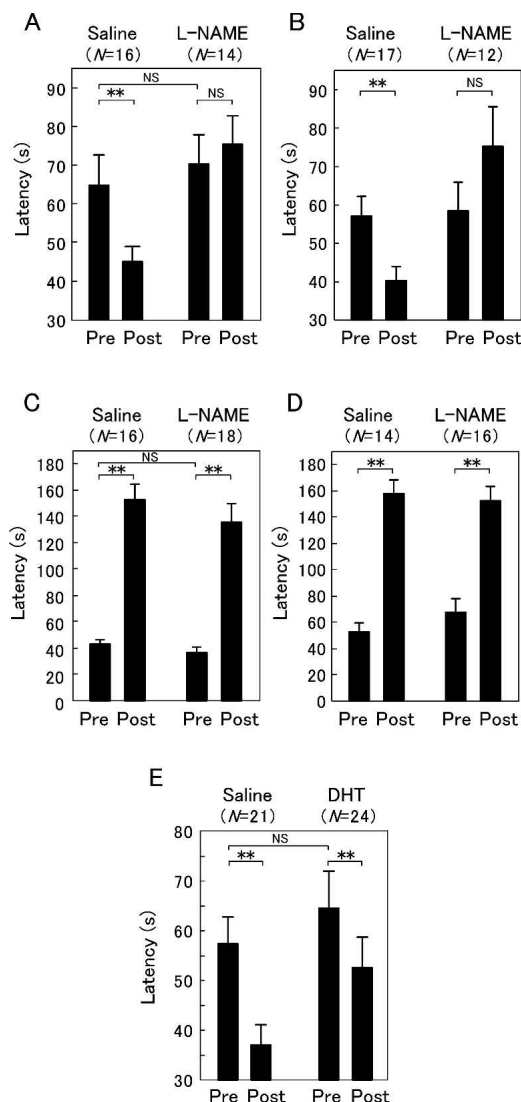


Figure 2. Effects of L-NAME and DHT on appetitive and aversive conditioning. (A) L-NAME injected before conditioning blocked the reduction in the post-conditioning latency in appetitive conditioning. In the slugs injected with saline 1 h before conditioning (left), the post-conditioning latency was significantly shorter than the preconditioning latency. The slugs injected with L-NAME (right) showed no significant reduction in the post-conditioning latency (Wilcoxon's signed ranks test, $**P < 0.01$, NS indicates not significant). The preconditioning latencies were not significantly different between the saline and L-NAME groups (Mann-Whitney *U*-test). (B) L-NAME injected before testing blocked the reduction in the post-conditioning latency in appetitive conditioning. In the slugs injected with saline 1 h before testing (left), the post-conditioning latency was significantly shorter than the preconditioning latency. The slugs injected with L-NAME (right) showed no significant reduction in the post-conditioning latency (Wilcoxon's signed ranks test, $**P < 0.01$, NS indicates not significant). (C, D) Effects of L-NAME on aversive conditioning. (C) L-NAME injected before conditioning did not significantly affect the increase in the post-conditioning latency in aversive conditioning. In the slugs injected with saline 1 h before conditioning (left), the post-conditioning latency was significantly longer than the preconditioning latency. In the slugs that were injected with L-NAME (right), the post-conditioning latency was also significantly longer than the preconditioning latency (Wilcoxon's signed ranks test, $**P < 0.01$). The preconditioning latencies were not significantly different between the saline and L-NAME groups (Mann-Whitney *U*-test). (D) L-NAME injected before testing did not affect the increase in the post-conditioning latency in aversive conditioning. In the slugs injected with saline 1 h before testing (left), the post-conditioning latency was significantly longer than the preconditioning latency. In the slugs injected with L-NAME (right), the post-conditioning latency was also significantly longer than the preconditioning latency (Wilcoxon's signed ranks test, $**P < 0.01$). (E) Effects of DHT on appetitive conditioning. In the slugs injected with saline 5 d before conditioning (left), the post-conditioning latency was significantly shorter than the preconditioning latency. In the slugs injected with DHT (right), the post-conditioning latency was also significantly shorter than the preconditioning latency (Wilcoxon's signed ranks test, $**P < 0.01$). The preconditioning latencies were not significantly different between the saline and DHT groups (Mann-Whitney *U*-test).

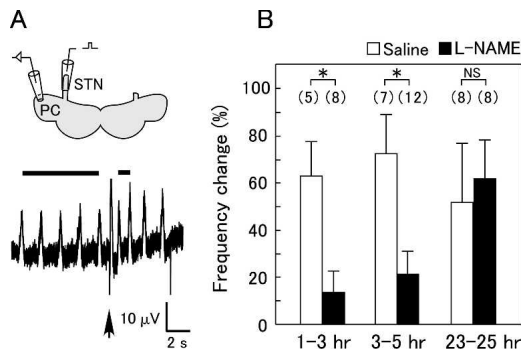


Figure 3. Effect of L-NAME on the LFP oscillation recorded in isolated brain preparations made at various times after injection. (A) The superior tentacle nerve (STN) was stimulated with a suction electrode, and the LFP was recorded from the PC. An example of LFP in an untreated slug is shown. A single electrical pulse was applied to the superior tentacle nerve at the arrow (0.5 V, 1-msec duration). This induces an increase in the LFP frequency. The stimulus-induced frequency change is calculated from the post-stimulus interval (right horizontal bar) and the average of the four prestimulus intervals (left horizontal bar). (B) Normalized frequency changes in preparations made at various times after injection of saline or L-NAME. Up to 5 h after injection, the frequency increase was significantly diminished by L-NAME, whereas at 23–25 h, there was no difference between the saline and L-NAME groups. The numbers above the bars indicate the number of samples. Student's *t*-test, **P* < 0.05.

Limax, DHT blocked the acquisition of memory but did not block retrieval (Shirahata et al. 2006). In others, acquisition and retrieval seem to share the same mechanisms. In the proboscis extension learning in the honeybee, for example, octopamine is necessary for both acquisition and retrieval (Farooqui et al. 2003), and the same reinforcing neuron is activated during both processes (Hammer 1993). The function of NO in the appetitive learning of *Limax* can be another example of common mechanisms for acquisition and retrieval.

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