In Vitro Odor-Aversion Conditioning in a Terrestrial Mollusk

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Running title

In vitro olfactory conditioning

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Abstract

We developed an *in vitro* odor-aversion conditioning system in the terrestrial mollusk, *Limax*, and found a behavioral correlate of network oscillation in the olfactory CNS. We first examined the odor-induced behavior of *Limax*, after odor-aversion conditioning *in vivo*. Shortening of mantle muscles was specifically observed in response to aversively-conditioned odors. We have previously identified that parietal nerves, which project to the mantle muscle in *Limax*, regulate shortening of the mantle muscle. We therefore isolated whole brains containing noses (sensory organs) and parietal nerves (motor output), and applied an odor-aversion conditioning paradigm to these *in vitro* preparations. Before the *in vitro* conditioning, application of attractive odors to the noses did not elicit any discharge in the parietal nerves. However, after odor-aversion conditioning, discharges in the parietal nerves were observed in response to the natively-attractive but aversively-conditioned odors. We also found that network oscillation frequency in the procerebrum (PC), the olfactory CNS of *Limax*, increased specifically in response to the aversively-conditioned odors that elicited avoidance behavior. In naïve (non-conditioned) preparations, increases in the PC oscillation frequency were observed specifically in response to innately-aversive odors. These results indicate that the isolated brains have an ability of odor-learning. They also suggest that changes in PC network oscillation are associated with aversively-conditioned and innately-aversive odors, both of which elicit avoidance behavior. This *in vitro* conditioning system would be an effective approach for exploring the neural mechanism to determine the aversion to odors.
Introduction

Network oscillations in olfactory systems are ubiquitous phenomena, and have been reported in a wide range of species from vertebrates to invertebrates (Adrian, 1950; Freeman, 1978; Gelperin and Tank, 1990; Laurent and Davidowitz, 1994; Laurent and Naraghi, 1994). Such network oscillations are generated by synchronous oscillation of electrical activities among numerous neurons in olfactory systems. The role of these network oscillations in olfactory information processing has been explored in invertebrate olfactory systems, especially in insect olfactory systems (see review; Laurent, 2002).

One important advantage of molluscan nervous systems is that molluscan brains in vitro retain several types of computational properties found in vivo (e.g., learning and memory and central pattern generator). These in vitro preparations enable us to conduct complicated electrophysiological recordings. The procerebrum (PC) is the olfactory center of Limax and related species (Chase, 1985). The PC consists of $10^5$ neurons and exhibits ongoing network oscillation even in vitro (Gelperin and Tank, 1990; Kleinfeld et al., 1994). The network oscillations are analogous to the network oscillation in vivo (Cooke and Gelperin, 2001). The network oscillations in the PC are modulated in response to odor application to the nose (Gelperin and Tank, 1990; Delaney et al., 1994; Kimura et al., 1998b). In addition, odor application to the noses produces discharges in efferent nerves of in vitro preparations (Gervais et al., 1996; Teyke and Gelperin, 1999; Inoue et al., 2004).

Limax has abilities of odor-learning in vivo (Sahley et al., 1981; Kimura et al., 1998a). However, it is still unclear whether abilities of odor-learning are preserved in vitro. Can isolated brains, containing the nose and motor output, acquire memories of odors? Several types of in vitro conditioning systems have been reported using other molluscan nervous systems: classical conditioning of the gill-withdrawal reflex system in Aplysia (Lukowiak and Sahley, 1981),
classical conditioning in feeding systems in *Limax* (Chang and Gelperin, 1980), *Lymnaea* (Kemenes et al., 1997), and *Aplysia* (Mozzachiodi et al., 2003; Reyes et al., 2005), and operant conditioning in feeding systems in *Aplysia* (Nargeot et al., 1997). However, there have been no reports of *in vitro* conditioning in olfactory systems. Such a system would enable us to study the role of network oscillation in olfactory information processing and olfactory learning. In this study, we report an ability of odor-learning in *in vitro* preparations of *Limax*. Using this *in vitro* odor-aversion conditioning system, we also show that network oscillation frequency in the PC increases specifically in response to aversively-conditioned and innately-aversive odors, both of which elicit avoidance behavior.
Materials and Methods

In vitro preparation and electrophysiology. We used laboratory bred slugs, Limax marginatus, whose body weight was 0.5-1.0 g. The brain, containing the nose (for application of conditioned stimulus [CS]), the lip and pedal nerves (for unconditioned stimulus [UCS] application) and the parietal nerve (for motor output observation), was isolated from each slug. The isolated brains were placed in physiological saline containing (in mM): 70 NaCl, 2 KCl, 4.7 MgCl₂, 4.9 CaCl₂, 5.0 glucose, 5.0 HEPES/Na, adjusted to pH 7.6 (Inoue et al., 2004). Extracellular recordings of the parietal nerve were made with a conventional glass suction electrode (150-1,000 Hz band-pass filtered), and local field potential (LFP) recordings in the PC were made with a fine glass electrode (0.08-30 Hz band-pass filtered). We waited for about 2 hours for recovery from dissection. All experiments were performed at room temperature (20-25°C).

In vitro odor-aversion conditioning. We used two natural odorants, carrot and cucumber, as CS odors. The two odorants are both natively attractive to Limax (Kimura et al., 1998a). The odors were delivered to the nose pad in air, using an odor stimulator (Inoue et al., 2004). For the UCS, two afferent nerves, the external lip nerve (ELn) and the anterior pedal nerve (APn), were electrically stimulated (5 V, 6.67 Hz, 15 s) using glass suction electrodes. Electrical stimulation of these nerves is an adequate UCS for in vitro conditioning of Limax, since Limax can be aversively conditioned by electrical stimulation of the anterior-dorsal parts of the body in vivo (Fig. 1). During aversive conditioning, one odor was paired with the UCS and the other odor was unpaired with the UCS in the same preparations (differential conditioning; see Fig. 2B). The paired odor was referred to as the CS⁺ and the unpaired odor was referred to as the CS⁻ in the text and figure legends. The conditioned response (CR) was quantified as the number of p-VN spikes, which were extracellularly recorded from the left parietal nerves. To identify p-VN spikes in the left parietal nerves, we touched the nose pad before pre-test period (see Fig. 2B), and observed
fully-evoked discharges in the left parietal nerves. These evoked discharges had larger amplitudes than spontaneous discharges, and the larger discharges were classified into several units (basically two units) based on the distinguishable amplitudes. A unit of maximum-amplitude spikes in the left parietal nerve was regarded as $p$-VN spikes in this study, as previously reported (Inoue et al., 2004).

Quantitative and statistical analysis. The normalized mantle length in Fig. 1C was calculated as previously described (Inoue et al., 2004). Briefly, after the conditioning, the mantle lengths (see Fig. 1B) at three arbitrary time points during spontaneous behavior were measured and averaged (defined as the averaged length). The mantle lengths just before and during odor presentation were then measured and normalized to the averaged length (defined as the normalized mantle length in Fig. 1C). In Fig. 2, spiking activity in the posterior viceral neuron ($p$-VN) was defined as the number of $p$-VN spikes during 15-s odor-stimulus periods followed by subtraction of the number of $p$-VN spikes during 15-s pre-odor periods. The index of learning (IOL) in Fig. 3 was defined as $(CS^+_\text{post} - CS^-\text{post}) - (CS^+\text{pre} - CS^-\text{pre})$, in which $CS^+_\text{post}$ or $CS^+\text{pre}$ ($CS^-\text{post}$ or $CS^-\text{pre}$) indicates the spiking activity of the $p$-VN in response to $CS^+$ odor ($CS^-$ odor) after or before the in vitro conditioning, respectively. Thus, a positive change in the IOL indicates that the isolated brains show odor aversion learning more clearly. All quantitative data were represented as mean ± SEM. For statistical analyses of the difference between before and after odor application, two-tailed paired $t$-tests were used (*$P < 0.05$, **$P < 0.01$, n.s. not significant).
Results

**In vivo odor-aversion conditioning in Limax**

*Limax* is known to have an ability of odor-learning (Sahley et al., 1981; Kimura et al., 1998a). After odor-aversion conditioning, *Limax* exhibits avoidance behavior in response to natively-attractive but aversively-conditioned odors. However, the neural components governing this odor-induced avoidance behavior remain unclear. Identification of the neural components is essential to reproducing odor-aversion conditioning *in vitro*. To identify the neural components, we first examined the muscle movement of *Limax* during odor-induced avoidance behavior *in vivo*.

We applied a differential conditioning paradigm, where two different odors (carrot and cucumber) were used. Since both odors are natively attractive odors for *Limax* (Kimura et al., 1998a), the animals approached these odorants (*Before conditioning* in Fig. 1A). During the differential conditioning, one odor (*CS+*) was paired with a noxious electrical stimulus (*UCS*; *During conditioning* in Fig. 1A), and then the other odor (*CS−*) alone was applied to the same animals one hour after CS+−UCS pairing. One hour is too long to pair the CS− and UCS. After the conditioning, they exhibited withdrawal behavior from the CS+ odorant, but still exhibited an approach behavior to the CS− odorant (*After conditioning* in Fig. 1A). During the withdrawal behavior from the CS+ odorant, shortening of the mantle muscle was observed (*Mantle* in Fig. 1B).

We quantitatively measured changes in the length of the mantle muscle in response to odors after the differential conditioning (Fig. 1C). After one time pairing, we applied the CS+ odor and CS− odor to each animal. In the cucumber-conditioned group (Fig. 1C; n = 5 slugs), the mantle shortening was observed in response to the CS+ cucumber odor (74.7 ± 3.4% of the control, n = 5; *P* < 0.01), but not in response to the CS− carrot odor (92.6 ± 7.5% of the control, n = 5; *P* >
0.05). By contrast, in the carrot-conditioned group (Fig. 1C; n = 5 slugs), the mantle shortening was observed in response to the CS$^+$ carrot odor (74.6 ± 8.4 % of the control, n = 5; $P < 0.05$), but not in response to the CS$^-$ cucumber odor (89.6 ± 12.1% of the control, n = 5; $P > 0.05$). These results indicate that shortening of the mantle muscle is associated with the avoidance behavior in response to aversively-conditioned odors.

The parietal nerves connect the mantle muscle to the brain, and carry motor and sensory information ($Pn$ in Fig. 1B). We previously reported that lesioning of the parietal nerves in vivo completely blocked repellent odor-induced shortening of the mantle muscle (Inoue et al., 2004), which indicates that the parietal nerves regulate odor-induced shortening of the mantle muscle. Discharges in the parietal nerves are spontaneously generated in vitro, but larger-amplitude discharges in the parietal nerves are observed in response to natively-aversive odors (Inoue et al., 2004). Such larger-amplitude discharges are not observed in response to attractive odors (Inoue et al., 2004). We therefore used the discharges in the parietal nerves, as an in vitro index of odor-induced avoidance behavior, in the following in vitro experiments (Figs. 2 – 4).

**In vitro odor-aversion conditioning using the isolated brain of Limax**

We next examined whether or not the nose-brain-parietal nerve preparations were aversively conditioned, using the in vitro index of avoidance behavior (Fig. 2). The nose-brain-parietal nerve preparations and stimulating/recording electrodes are shown in Fig. 2A. The conditioning paradigm in vitro (Fig. 2B) is a differential conditioning paradigm similar to that used in vivo, except that the experiments in Fig. 2 were performed using isolated brains. One advantage of this conditioning paradigm is that both paired and unpaired odors can be tested in the same in vitro preparation.

In the isolated preparations, attractive odors (cucumber and carrot) elicited no change in
discharges of the parietal nerve (1st and 2nd traces in Fig. 2C). However, after one time pairing between the attractive cucumber odor (paired odor; CS+) and electrical stimulation of ELn and APn (UCS), application of the same cucumber odor elicited larger-amplitude discharges in the parietal nerve (3rd and 5th traces in Fig. 2C). Unpaired carrot odor (CS-) did not elicit the discharges (4th and 6th traces in Fig. 2C). Thus, the isolated brain selectively produces the large-amplitude discharges in the parietal nerve (i.e., an in vitro index of avoidance behavior) in response to the aversively-conditioned cucumber odor.

To quantify the effect of the in vitro odor-aversion conditioning, we counted the spikes in the p-VN, which is a motoneuron projecting its axon into the parietal nerves and innervating the mantle muscle (Inoue et al., 2004). The p-VN spikes could be readily identified by extracellular recording of the left parietal nerves (indicated by horizontal arrowheads in Fig. 2C; see Materials and Methods). In the cucumber-conditioned brains (Fig. 2D), the spiking activity in the p-VN increased in response to the cucumber odor (spiking activity before conditioning→spiking activity after conditioning; 0.41 ± 0.22→3.35 ± 1.02, n = 17; \( P < 0.01 \)), whereas the spiking activity did not change in response to the carrot odor (0.41 ± 0.25→0.53 ± 0.28, n = 17; \( P > 0.05 \)).

To establish whether the changes in spiking activity were indeed conditioning-dependent, a control experiment was performed. In this experiment, we used carrot odor as the CS+ (paired) odor, and cucumber odor as the CS- (unpaired) odor. In the carrot-conditioned group (Fig. 2D), the spiking activity increased in response to the paired carrot odor (0.35 ± 0.15→2.53 ± 0.66, n = 17; \( P < 0.01 \)), whereas the spiking activity did not change in response to the unpaired cucumber odor (0.18 ± 0.10→0.53 ± 0.28, n = 17; \( P > 0.05 \)). These results indicate that the nose-brain-parietal nerve preparations could be aversively conditioned, using an in vitro index of avoidance behavior.
Changes in the PC network oscillation after *in vitro* odor-aversion conditioning

The PC consists of $10^5$ neurons, and this large population of neurons spontaneously exhibits regular synchronized oscillation at about 0.7 Hz (Gelperin and Tank, 1990). The synchronized oscillation can be recorded as oscillations of the local field potential (LFP) in the PC (Gelperin and Tank, 1990). We performed simultaneous recordings of discharges in the $p$-VN and LFP oscillations in the PC (Fig. 3).

As shown in Fig. 2C, application of attractive cucumber and carrot odors to the nose hardly changed the pattern of discharges in the $p$-VN. LFP oscillations in the PC also hardly changed in response to these attractive odors (*Before conditioning* in Fig. 3A and 3B). However, after the cucumber odor was paired with the UCS, application of the cucumber odor increased the PC oscillation frequency approximately 2-fold (*After conditioning* in Fig. 3A and 3B), as well as elicited $p$-VN spikes (indicated by horizontal arrowheads in Fig. 3A). The unpaired carrot odor did not change the PC oscillation frequency even after conditioning. We then quantitatively analyzed the odor-evoked change in the LFP frequency, in a series of the *in vitro* odor-aversion conditioning experiments (Fig. 3C). After the *in vitro* conditioning, the aversively-conditioned odors selectively increased the frequency of LFP oscillation (*CS*+ *after conditioning*, 127.1 ± 8.2% of the baseline frequency, n = 20; $P < 0.01$). The frequency of LFP oscillation was not modulated by the unpaired odors (*CS*− *after conditioning*, 104.4 ± 2.9% of the baseline frequency, n = 20; $P > 0.05$).

We then studied the relationship between the conditioned odor-evoked increase in the PC oscillation frequency and the index of learning (IOL; see Materials and Methods). Figure 3D shows plots of the change in the conditioned odor-evoked LFP frequency, against the IOL (n = 20). We observed a moderate positive correlation, where the correlation coefficient was 0.57 (Fig. 3D).
In Fig. 3E, we classified the conditioned isolated-brains (n = 20) into good-learner (n = 7/20) and poor-learner (n = 13/20) preparations, based on the IOL. The IOL classification revealed that the increase in PC oscillation frequency after conditioning was only observed in the good-learner group (Fig. 3E; 153.9 ± 15.9% after conditioning; n = 7, \( P < 0.05 \)), but not in the poor-learner group (Fig. 3E; 112.7 ± 7.1% after conditioning; n = 13, \( P > 0.05 \)). These results clearly show that the frequency of the PC network oscillation is correlated with discharges in the \( p-VN \), an in vitro index of odor-avoidance behavior.

**Changes in the PC oscillation frequency in response to natively-aversive odors**

There are two possible explanations for the selective increase in PC oscillation frequency in response to aversively-conditioned odors. One is that the increase is correlated with the aversion to the applied odor, and the other is that the increase is correlated with the learning of the applied odor. To address this issue, we applied two types of natively-aversive odors (onion and garlic) and two types of natively-attractive odors (potato and rat chow) to naive preparations that had not received any conditioning (Fig. 4).

We previously showed that mantle shortening of *Limax* in vivo was selectively induced by the two natively-aversive odors, onion and garlic (Inoue et al., 2004). Our in vitro study has also revealed that spikes in the \( p-VN \) were generated by the two aversive odors, but were not induced by the two natively-attractive odors (Inoue et al., 2004; also see \( Pn \) in Fig. 4A). As shown in Fig. 4A in which onion and potato odors were applied to a naive preparation, the PC oscillation frequency increased specifically in response to the aversive onion odor. Quantitative analysis (Fig. 4B; n = 5) revealed that the PC oscillation frequency increased in response to the aversive onion odor (149.5 ± 14.8% of the baseline frequency, n = 5; \( P < 0.05 \)), but hardly increased in response to the attractive potato odor (111.1 ± 7.4% of the baseline frequency, n = 5; \( P > 0.05 \)).
In another set of preparations to which garlic and rat chow odors were applied (Fig. 4B; n = 5), the PC oscillation frequency increased in response to the aversive garlic odor (137.1 ± 11.1\% of the baseline frequency, n = 5; \( P < 0.05 \)), but hardly increased in response to the attractive rat chow odor (108.8 ± 6.5\% of the baseline frequency, n = 5; \( P > 0.05 \)). These results indicate that odor-induced increases in PC oscillation frequency are correlated with the aversion to the applied odor.
Discussion

In this study, we developed an \textit{in vitro} odor-aversion conditioning system in \textit{Limax} (Figs. 1 and 2). We found that, once attractive odors were paired with a noxious electrical stimulus \textit{in vitro}, the attractive odors produced discharges in the parietal nerve and the $p$-VN, an \textit{in vitro} index of odor-avoidance behavior. We also found that the frequency of PC network oscillation increased specifically in response to aversively-conditioned odors, which elicited the discharges in the parietal nerve and the $p$-VN (Fig. 3). We finally found that the PC oscillation frequency also increased in response to natively-aversive odors (Fig. 4).

Molluscan nervous systems are useful preparations for studying the dynamics and function of neuronal circuits, because molluscan brains containing sensory and motor organs can carry out several types of computations, such as learning and memory, even in artificial ringer solution. To study the mechanism of learning and memory at the circuit level, several types of \textit{in vitro} conditioning systems have been developed: e.g., classical conditioning of the gill withdrawal reflex in \textit{Aplysia} (Lukowiak and Sahley, 1981); classical conditioning in the feeding system of \textit{Limax} (Chang and Gelperin, 1980), \textit{Lymnaea} (Kemenes et al., 1997), and \textit{Aplysia} (Mozzachiodi et al., 2003; Reyes et al., 2005); and operant conditioning in the feeding system of \textit{Aplysia} (Nargeot et al., 1997). The study on the gill-withdrawal system has focused on conditioning-dependent synaptic changes, and the studies on the feeding system have also focused on conditioning-dependent changes in central pattern generator circuits. In this study, we reported conditioning-dependent changes in network oscillations in olfactory systems, using the \textit{in vitro} odor-aversion conditioning system.

The PC exhibits ongoing network oscillations \textit{in vitro} (Gelperin and Tank, 1990; Kleinfeld et al., 1994) and \textit{in vivo} (Cooke and Gelperin, 2001). In \textit{in vitro} preparations, it has been shown that application of odors to the nose modulates the frequency of PC network oscillations
(Gelperin and Tank, 1990; Gervais et al., 1996; Kimura et al., 1998b). These studies have shown that attractive odors and aversive odors exhibit distinct patterns of change in the PC oscillation frequency. However, it has been unknown whether different patterns of frequency change are coupled with different patterns of behavioral output. In this study, we found that oscillation frequency in the PC increased specifically in response to aversively-conditioned odors (Fig. 3C). We then found that the increase in PC oscillation frequency was correlated with discharges in the $p$-VN, an in vitro index of odor-avoidance behavior (Fig. 3D and 3E). We also found that, in naive preparations, increases in PC oscillation frequency were observed in response to natively-aversive odors but not to natively-attractive ones (Fig. 4). Thus, changes in PC oscillation frequency are associated specifically with odors that elicit avoidance behavior, both aversively-conditioned odors and natively-aversive odors.

Although our experiments showed that increases in PC oscillation frequency were strongly correlated with the aversion to the applied odors, we could not directly demonstrate a causal relationship by lesioning of the PC. This is partially due to a technical difficulty: we should maintain the viability of the in vitro preparations throughout the series of in vitro conditioning experiments (at least 5 hours after dissection). However, odor-induced neural responses were progressively reduced by lesioning of the PC or removal of sheaths surrounding the PC. Thus it will be necessary to develop more viable in vitro preparations, in order to directly examine the causal relationship.

Studies on the insect olfactory system have revealed that discrimination of closely related odors is impaired by blockage of synchronized activity in the antennal lobe (Stopfer et al., 1997; MacLeod et al., 1998). Network oscillation in the PC is also involved in discriminating closely related odors in Limax (Teyke and Gelperin, 1999). In this study, we propose that network oscillation in the PC is involved in the aversion of odors. Thus, it is assumed that the PC network
oscillation is involved in different types of olfactory processing, discrimination between closely related odors and discrimination between aversive and attractive odors. It will be interesting to explore the neural mechanism underlying the different types of olfactory processing.

Then, how does the increase in PC oscillation frequency generate discharges in the p-VN? We previously found an identifiable serotonergic neuron, the *posterior cerebral serotonergic cell* (p-CSC; Inoue et al., 2004). Discharges in the p-CSC released serotonin in the tentacle ganglion (TG). Serotonin in the TG suppressed aversive odor-induced discharges in the p-VN (Inoue et al., 2004). These previous findings suggest that the p-CSC may be a neuron that regulates odor-induced p-VN discharges. Our preliminary studies revealed that pharmacological manipulation of the PC modulated the firing rate of the p-CSC; increase of PC oscillation frequency by acetylcholine (Watanabe et al., 2001) decreased the p-CSC firing rate (Inoue et al., unpublished observations). These preliminary studies suggest that there may be a pathway from the PC to the p-CSC. We have not yet succeeded in making intracellular recordings of the p-CSC throughout the *in vitro* conditioning paradigm. The approach will enable us to directly examine the neural mechanism that the oscillation frequency in the PC mediates odor-guided avoidance behavior.
Acknowledgements

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References


23. **Stopfer M, Bhagavan S, Smith BH, and Laurent G.** Impaired odour discrimination on


Figure legends

Figure 1. In vivo odor-aversion conditioning in Limax

(A) The paradigm of odor-aversion conditioning (differential conditioning) in vivo. The withdrawal (escape) behavior in response to a paired odor (CS⁺ odor after conditioning) was accompanied by shortening of the mantle muscle, which is located at the back of Limax (indicated by brown color). The approach behavior was observed in response to an unpaired odor (CS⁻ odor after conditioning).

(B) Structure of Limax. Odors (conditioned stimulus; CS) are received at the nose (located at the tip of the tentacles (T)), and are then transmitted to the brain via the olfactory nerve (On) in the tentacle. The olfactory nerve is directly connected to the procerebrum (PC) in the brain. The unconditioned stimulus (UCS) was electrically applied to the anterior-dorsal part of the body. The external lip nerve (ELn) and anterior pedal nerve (APn) connect the anterior-dorsal part of the body to the brain. The parietal nerves (Pn) are involved in shortening of the mantle muscle (Mantle), which is a component of Limax’s escape behavior.

(C) Summary of odor-induced mantle shortening after conditioning (cucumber-conditioned group, n = 5; carrot-conditioned group, n = 5). The normalized mantle length was calculated by dividing the mantle length just before or during odor presentation by the mantle length during spontaneous behavior. Open or filled columns indicate the normalized mantle length just before or during odor presentation, respectively. Mantle shortening was selectively observed in response to conditioned CS⁺ odors.

Figure 2. In vitro odor-aversion conditioning using the isolated brain of Limax

(A) Schematic drawing of the left hemisphere of the isolated brain of Limax (cf. Fig. 1B). p-VN, posterior visceral neuron.
Experimental paradigm of in vitro odor-aversion conditioning. Before conditioning, two odors (CS\(^+\) and CS\(^-\)) were applied for 15 s. The interval between two stimuli was 30 min. During the period of conditioning, CS\(^+\) odors (20 s) were paired with an electrical stimulus (UCS), whereas CS\(^-\) odors (20 s) were applied 30 min after the conditioning (i.e., backward conditioning). Since the 30 min was too long to pair the CS\(^-\) and UCS, we regarded the CS\(^-\) odor as unpaired odors. One hour after conditioning, we restarted to apply the CS\(^+\) and CS\(^-\) odors (15 s), where the interval of two stimuli was 30 min.

Discharges in the parietal nerve in response to odors, in a series of odor-aversion differential conditioning experiments. Underlines indicate odor-stimulus period (15 s) and horizontal arrowheads indicate discharges of the identified motoneuron, the \(p\)-VN. 0 h is the time when the CS\(^+\) and UCS were paired. After conditioning in vitro, discharges in the \(p\)-VN were selectively induced in response to CS\(^+\) (paired) odors. Scale bars are 4 s and 0.1 mV.

Summary of spiking activity in the \(p\)-VN, in the cucumber-conditioned group (n = 17) and carrot-conditioned group (n = 17). The spiking activity was defined as the number of odor-induced \(p\)-VN spikes minus the number of spontaneous \(p\)-VN spikes before odor presentation. Open or filled columns indicate spiking activity in the \(p\)-VN before or after conditioning, respectively.

Figure 3. Changes in the PC network oscillation after in vitro odor-aversion conditioning

Simultaneous recordings of discharges in the parietal nerve (\(Pn\); motor output) and the LFP oscillations in the PC (\(PC\); olfactory CNS activity) in a cucumber-conditioned preparation. Underlines indicate odor-presentation period (15 s) and horizontal arrowheads indicate \(p\)-VN discharges. Scale bars are 6 s, 12.5 \(\mu\)V (\(PC\)), and 0.1 mV (\(Pn\)).

Plots of instantaneous LFP frequencies in the preparation of Fig. 3A, represented as a
function of time. The instantaneous LFP frequencies (Hz) are the inverse of the time between neighboring positive peaks of cyclic LFP oscillation. Filled or open circles indicate the responses to CS⁺ (cucumber) or CS⁻ (carrot) odors, respectively.

(C) Summary data (n = 20; 11 carrot-conditioned brains and 9 cucumber-conditioned brains) showing the averaged LFP frequencies in the odor-stimulus period normalized by those in the pre-odor period (% change). Note that LFP oscillation frequency increased only in response to the CS⁺ odor after the odor-aversion conditioning.

(D) Plots of the change in PC oscillation frequency in response to aversively-conditioned odors (i.e., CS⁺ after conditioning in Fig. 3C), against the IOL (n = 20). The correlation coefficient was 0.57.

(E) The conditioning-dependent change in PC oscillation frequency was remarkably observed in the good-learner group. All aversively-conditioned preparations (n = 20) were classified into good-learners (n = 7/20) or poor-learners (n = 13/20), based on the IOL. In this analysis, we regarded preparations with IOL ≥ 3 as good-learners, and preparations with IOL < 3 as poor-learners. Open or filled columns indicate averaged LFP frequencies in CS⁺ odor-stimulus periods normalized by those in the pre-odor period (% change), before or after the conditioning, respectively.

Figure 4. Change in the PC network oscillation in response to natively-aversive odors

(A) Simultaneous recordings of discharges in the parietal nerve (Pn) and the LFP oscillations in the PC (PC) in a naive preparation that had not received any conditioning. Natively-aversive onion odor and natively-attractive potato odor were applied for 30 s (indicated by underlines). Horizontal arrowhead indicates $p$-VN discharges. The PC oscillation frequency increased strikingly in response to the onion odor. Scale bars are 5 s, 15 µV (PC), and 0.2
mV ($Pn$).

(B) Summary data showing the averaged LFP frequencies in the 30-s odor-stimulus period normalized by those in the 30-s pre-odor period (% change). Two sets of *in vitro* preparations were made ($n = 5$ in each set). Onion and potato odors were applied to one set of preparations ($n = 5$), and garlic and rat chow odors were applied to the other set ($n = 5$).
Fig. 1

A

Before conditioning

↓

During conditioning

↓

After conditioning

CS' odor

Cucumber

Electrical stimulus (UCS)

CS' odor

Carrot

(Cuttle behavior)

(Approach behavior)

B

C

Olfactory conditioned

 normalized response length

60

50

40

30

20

10

0

Normalized response length (%)
Fig. 2

A

B

C

Cucumber-conditioned

Cucumber (-1 h)

Carrot (-0.5 h)

Conditioning

Cucumber (+1 h)

Carrot (+1.5 h)

Carrot (+2 h)

Carrot (+2.5 h)

D

Cucumber-conditioned

Carrot-conditioned

Sucking activity

Cucumber (CS')

Carrot (CS')

Cucumber (CS')

Carrot (CS')
Fig. 4

A

Onion

PC

Pn

Potato

PC

Pn

B

Odor-induced LP frequency change (%)

Onion  Potato  Grillo  Red chile

n.s.  n.s.  n.s.