In Vitro Study of Odor-Evoked Behavior in a Terrestrial Mollusk

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Inoue, Tsuoshi, Yasuko Inokuma, Satoshi Watanabe, and Yutaka Kirino. In vitro study of odor-evoked behavior in a terrestrial mollusk. J Neurophysiol 91: 372–381, 2004; First published September 17, 2003; 10.1152/jn.00247.2003. To explore neural mechanisms how olfactory information is processed in the brain and finally converted into behavior, it would be useful to have isolated whole brains that include both olfactory organs and motor output. In the present study, we identified an in vitro index of odor-evoked behavior in the terrestrial mollusk Limax and also studied the modulation of this in vitro index of the behavior. We determined that shortening of the mantle muscles is one of the withdrawal responses selectively induced by aversive odors and that the shortening is mediated by a pair of parietal nerves. We also identified a motoneuron (named the posterior visceral neuron, p-VN) that projects to the parietal nerve and innervates the mantle muscles. When we applied various odors to the nose in vivo, the isolated molluscan brains, only aversive odors induced discharges in the p-VN. These results indicate that p-VN discharges can serve as an in vitro index of odor-induced aversive behavior. We also identified a novel serotonergic neuron (named the posterior cerebral serotonergic cell, p-CSC). Discharges in the p-CSC released serotonin to the tentacle ganglion (TG); serotonin in the TG then inhibited odor-induced discharges in the p-VN, the in vitro index of aversive behavior. These results suggest that the serotonergic system is involved in the regulation of approach and avoidance behavior in Limax.

INTRODUCTION

Olfactory signals are first received in the nose, processed in the brain, and finally converted into behavior. How is the odor-guided behavior regulated? To explore the neural mechanisms, clarification of the neural pathway from the olfactory organs to behavioral output is prerequisite. Because understanding such neural pathways remains elusive in the mammalian brain, we used an in vitro nervous system of the terrestrial mollusk, Limax. An advantage of using Limax is that molluscan nervous systems retain their function in the isolated brain, which can survive for a long period of time in physiological saline. The in vitro preparation enables us to easily employ electrophysiological means for detailed study on the nervous system.

Limax has highly developed abilities of odor discrimination and odor learning (Sahley et al. 1981a,b). Thus Limax can discriminate various odors and select attractive or aversive behavior in response to the odors. Procererebrum (PC) is known to be the olfactory center of Limax (Chase 1985; Kimura et al. 1998a). There are many excellent studies focusing on the PC itself. The PC has been investigated at synaptic level (Inoue et al. 2000; Kleinfeld et al. 1994) and at network level (Cook et al. 2000; Kleinfeld et al. 1994) and at network level (Cooke 1994; Kimura et al. 1998a). There are many excellent studies focusing on the PC itself. The PC has been investigated at synaptic level (Inoue et al. 2000; Kleinfeld et al. 1994) and at network level (Cook et al. 2000; Kleinfeld et al. 1994). Thus PC itself. The PC has been investigated at synaptic level (Inoue et al. 2000; Kleinfeld et al. 1994) and at network level (Cook et al. 2000; Kleinfeld et al. 1994). Thus PC itself. The PC has been investigated at synaptic level (Inoue et al. 2000; Kleinfeld et al. 1994) and at network level (Cook et al. 2000; Kleinfeld et al. 1994).

METHODS

Behavioral analysis

Throughout the present study, we used slugs, Limax marginatus, whose body weights were ~0.5–2 g. The slugs were placed on a glass plate that was laid on a sheet of graph paper with 1-mm grids. After the animals had calmed down and crawled straight, a smooth paste of odorants was applied as a half circle ahead of them. For measurement of the mantle shortening, we made a mark on the mantle ~1 cm away from the mantle tip, and we defined the length from the mantle tip to the mark as the mantle length (Fig. 1, A and B). We recorded the slug’s behavior with an 8-mm video tape recorder.

For the experiments shown in Fig. 1D, the parietal nerves were sectioned under anesthesia as follows. An isotonic Mg2+ solution was injected into the body cavity at ~0.5 ml/g body wt, and 5–10 min later the slug’s foot and mantle were pinned to a dish lined with silicone and Gelperin 2001; Gelperin and Tank 1990). The properties of the PC resemble those of both the mammalian olfactory bulb and the insect antennal lobe. However, there are a few studies focusing on the regions outside the PC using in vitro preparations. Although some follower neurons of the PC have been identified (Chase and Tolloczko 1989; Gelperin and Flores 1997; Shimozono et al. 2001), there is no report that these neurons actually regulate behavioral outputs. Some efferent nerves are responsive to odor-input in vitro (Gervais et al. 1996; Teyke and Gelperin 1999), but the exact correspondence between the odor-guided behavior in vivo and the odor-induced discharges in the nerves in vitro has not been clearly shown. Our question is how the behavioral change of Limax in response to odor input is regulated. Identification of the odor-evoked motor output pathway in vitro, finally including the PC, will lead directly to elucidation of this issue.

In the present study, we describe an in vitro index of odor-evoked aversive behavior in Limax. We focused on the behavior of Limax in response to innately attractive and aversive odors. We first identified a motoneuron, the posterior visceral neuron (p-VN), which is involved in an aversive behavior. We next examine changes in the p-VN activity in response to innately attractive and aversive odors and found the p-VN activity can be used as an in vitro index of odor-evoked aversive behavior. We finally show that the in vitro index of the aversive behavior is regulated by a serotonergic system in Limax. These results in the present study suggest that the aversive behavior is switched to the attractive behavior when the serotonergic system is activated.

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elastomer (Sylgard). Because the parietal nerves pass just beneath the back skin and descend to the base of the mantle, they were severed bilaterally through a hole we opened in the back skin, ~1–2 mm in diameter. Thereafter, the slugs were bathed for 5 min four times every 30 min with physiological saline at 4°C to remove Mg$^{2+}$ from their bodies and then 5–7 h later were behaviorally tested as in the preceding text. In parietal nerve-lesioned animals, spontaneous and odor-evoked behaviors were almost normal except for the disappearance of mantle contraction. After behavioral testing, the slugs were dissected to confirm that the lesion procedure had been successful.

In vitro preparation and electrophysiology

Each slug was anesthetized with an injection of isotonic Mg$^{2+}$ solution, and then its head was quickly removed. The head was placed in a dissection solution that was maintained at 4°C; the composition of the dissection solution was (in mM) 35.0 NaCl, 2.0 KCl, 28.0 MgCl$_2$, 4.9 CaCl$_2$, 5.0 glucose, and 5.0 HEPEIS/Na (adjusted to pH 7.6). Its composition was designed to increase MgCl$_2$ concentration sixfold and decrease Na concentration to maintain osmolarity from the composition of a physiological saline described in the following text. The circumesophageal ganglia with the posterior tentacles and parietal nerves attached bilaterally were isolated from the head. The isolated ganglia were transferred into a recording chamber filled with a physiological saline; the composition of the saline was (in mM) 70.0 NaCl, 2.0 KCl, 4.7 MgCl$_2$, 4.9 CaCl$_2$, 5.0 glucose, and 5.0 HEPEIS/Na (adjusted to pH 7.6). To record the excitatory junctional potential (EJP), the mantle-attached circumesophageal ganglion was isolated. The structure of the circumesophageal ganglion and the peripheral nerves is illustrated in Fig. 2B (Chang et al. 1974).

The discharges in the parietal nerves and EJPs in the mantle muscle were recorded extracellularly with conventional glass suction electrodes filled with the physiological saline. The electrical signals were band-pass-filtered between 150 and 1,000 Hz for the parietal nerve discharges and between 15 and 1,000 Hz for the EJP. Intracellular recording of the p-VN and the posterior cerebral serotonergic cell (p-CSC) was performed with a DC-coupled amplifier that was low-pass filtered at 3 kHz. The microelectrodes for intracellular recordings contained 3 M KCl and had resistances of 15–25 MΩ. Synchronous oscillation of the membrane potential of the tentacle ganglion (TG) neurons (Inokuma et al. 2002) can be recorded as an oscillation in the local field potential (LFP). The LFPs were recorded extracellularly using a glass pipette with a tip diameter of 20–50 µm. The LFP signal was band-pass filtered between 0.08 and 30 Hz. All experiments were performed at room temperature (20–25°C).

Odor and electrical stimulation

We used two odor-delivery systems in this study. For the experiments in Figs. 2 and 3, the recording chamber was divided with petroleum jelly (Vaseline) into two compartments for odor stimulation (see Fig. 2B). The nose (olfactory receptors) was laid in one compartment, and the circumesophageal ganglion was placed in the other compartment (Fig. 2B). In the compartment containing the nose, the physiological saline was drained and the nose was exposed to moistened air to enable application of airborne odors. For application of odors to the nose in Figs. 2 and 3, we used odorized air as a delivery system as described by Kimura et al. (1998b). Briefly, compressed air was deodorized through activated charcoal and moistened through water. This deodorized and moistened air was continuously applied to the odor pathway with a pair of electrical valves. The tip of the air puffer pipette was positioned ~3 mm away from the surface of the nose. Five odorants were used (rat chow, potato, and cucumber as attractants, garlic and onion as repellents).

In Fig. 6, we used another delivery system (an odorant solution), in which the chemical odorant amyl acetate (AA; 0.1% diluted by physiological saline) was applied from a fine puffer pipette (~5 μm of the tip diameter). Because the tip size of the puffer pipette was much smaller than the nose and held away from the surface of the nose, the AA solution may have been more diffused and diluted at the surface of the nose. Application of the AA is probably selective to the nose, and it is unlikely for the AA to diffuse to the circumesophageal ganglia (the reason is described in Application of serotonin using a fine puffing pipette). The advantage of this odor-delivery system is that we can perform odor-stimulation under conditions in which the nose...
and TG are submerged in the physiological saline. The TG is located close to the nose, but the TG in vivo is normally protected by the tentacle muscle. However, we had to puff serotonin directly to the TG and so had to remove the tentacle muscle surrounding the TG (Fig. 6). Air-exposure of the nose for odorized air-stimulation (as shown in Fig. 2B) was inevitably accompanied by air exposure of the bare TG. We found that air exposure of the bare TG as well as the nose resulted in severe damage to the TG. This damage could be avoided if we used the diluted AA as the odorant solution for odor stimulation because the TG was fully submerged in the physiological saline. Therefore we selected the delivery system by odorant solution seen in Fig. 6.

The posterior olfactory nerves were stimulated electrically via a conventional suction pipette.

**Application of serotonin using a fine puffing pipette**

In Figs. 5 and 6, we puffed serotonin (Wako Chemicals, Osaka, Japan) to the TG using a fine glass pipette (≈5 μm of tip diameter), to investigate the effect on odor-induced discharges in the p-VN. The TG is connected to the rest of the *Limon* brain via a long olfactory nerve, and thus the TG is located far away from the rest of the brain (see Figs. 5A and 6A). In addition, we used a fine glass pipette for the puffing and perfused the recording chamber continuously with the physiological saline. These features make possible selective application of serotonin to the TG. Actually, when we puffed serotonin to the TG using the fine glass pipette, we could not observe any change in the p-VN activity (see Fig. 6Ci, Pn). On the other hand, when we puffed serotonin directly to the circumesophageal ganglia, spikes in the p-VN were generated (data not shown). Thus serotonin puffed to the TG is not likely diffused to the circumesophageal ganglia. About the AA, we used same size of the fine glass pipette for puffing the odorant (≈5 μm of tip diameter) to the nose, and the nose is located far away from the circumesophageal ganglia (see Fig. 6A). Thus it is also likely that the AA puffed to the nose is not diffused to the circumesophageal ganglia. We filled the puffing pipette with 1 mM serotonin. However, the very small tip diameter and the perfusion of the recording chamber with the physiological saline are assumed to have reduced the actual concentration at the TG neurons, although we cannot estimate the actual concentration.

![Image](http://jn.physiology.org/)

**FIG. 2.** Identification of a motoneuron (p-VN) that innervates the mantle muscle. A: location and arborization pattern of the p-VN, visualized by intracellular injection of the fluorescent dye Lucifer yellow. This photograph contains only the abdominal ganglion and its peripheral nerves. The p-VN was located in the posterior region of the abdominal ganglion and had a soma of ≈80 μm in diameter. The p-VN extended its axon bilaterally into the parietal nerves. LpN, left parietal nerve; RpN, right parietal nerve. Scale bar is 100 μm. B: schematic drawing of the in vitro preparation of *Limax* and the recording chamber, used in Figs. 2 and 3. The CNS consists of the cerebral ganglion (CG), abdominal ganglion (AG), and pedal ganglion (PG). The On connects the nose with the CG. The Pu connects the AG to the mantle muscle. Filled circle, the posterior visceral neuron (p-VN) that is described in the present paper. The recording chamber is divided into 2 compartments; the nose lies in one compartment and the CNS in the other. The nose is exposed to air, and odorized air can be applied to the nose. In contrast, the compartment containing the CNS is submerged in physiological saline. Triangles mark the position of the intracellular recording electrode (open triangle) on the p-VN and extracellular recording electrodes (filled triangles) on the Pu and mantle muscle. C: electrical properties of the parietal nerve and p-VN. i: the parietal nerve spontaneously exhibited discharges with small amplitude (indicated by the horizontal arrow). The p-VN did not normally exhibit spikes. When the spikes in the p-VN were elicited by current injection, the spikes were propagated into the parietal nerve and the amplitude of the p-VN spike was much larger at the parietal nerve (indicated by horizontal arrowhead). Scale bars are 1 s and 0.15 (Pn) or 20 mV (p-VN). ii: this recording was derived from the same preparation used for current injection. The parietal nerve spontaneously exhibited discharges with small amplitude (indicated by the horizontal arrow). Electrical stimulation of the olfactory nerve (single pulse of 1-ms duration, 3 V) was applied at the time point indicated by the vertical arrowhead. The electrical stimulation depolarized the p-VN, which was followed by the action potentials (n = 9). These action potentials had a 1-to-1 correspondence with the maximal-amplitude spikes of the left parietal nerve (indicated by the horizontal arrowhead), recorded with conventional suction electrodes, in all cases (n = 99). Scale bars are 0.5 s, and 0.15 (Pn) or 20 mV (p-VN). D: stimulation by garlic odor to the nose excited the p-VN and elicited spikes in the p-VN. The garlic odor was applied at the underline. Scale bars are 5 s and 20 mV. E: excitatory junctional potentials (EJPs) in the mantle. The action potentials, induced by a 1-nA positive current injection into the p-VN, produced 1-for-1 EJPs at the mantle muscle. Horizontal scale bar is 250 ms, and vertical ones are 20 μV (EJPs), 0.2 (Pn) or 20 mV (p-VN). F: overtraced EJPs based on the peak of p-VN spikes that was derived from E. The latencies between the peak of p-VN spikes and onset of EJPs were constant. Horizontal scale bar is 10 ms, and vertical ones are 20 μV (EJPs), 0.2 (Pn) or 20 mV.
Morphology of the p-VN and the p-CSC

For visualization of the p-VN, we injected 3% Lucifer yellow CH (Sigma) as a fluorescent marker. For easy impalement with a microelectrode, we pretreated the isolated Limax brain with a protease (type IX; Sigma). After the neuron was impaled, the dye was injected iontophoretically using hyperpolarizing DC current pulses of 5–10 nA and 500-ms duration at 1 Hz for 15–20 min. After injection, the dye-filled ganglion was kept at 4°C for ~1 h to permit the dye to diffuse throughout the neurites of the p-VN, fixed for 1 h in 10% neutral buffered formalin (Wako Chemicals), dehydrated in a graded series of alcohols, and cleared in methyl salicylate. The fluorescence was excited and detected using a xenon lamp and a mirror unit equipped with an excitation filter [425 ± 22.5 nm], a dichroic mirror (475 nm), and an emission filter (535 ± 27.5 nm).

For visualization of the p-CSC, we injected 1.5% biocytin (Sigma) into the p-CSC for 45 min and fixed it for 1 h in 10% formalin. After fixation, the filled ganglion was put into 3% H2O2 for 30 min to reduce endogenous peroxidase activity and then transferred into an avidin-biotin complex solution (Vectastain Elite ABC kit, Vector) overnight at 4°C. The ganglion was visualized by 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan), dehydrated in a graded series of alcohols, and cleared in methyl salicylate. The morphology of the p-CSC was observed by conventional light microscopy and was drawn using a camera lucida.

Glyoxylic acid staining

Glyoxylic acid staining is typically used for visualizing monoamine-containing neurons (Kemenes et al. 1989; Lent 1982). A desheathed ganglion of Limax was immersed in 220 mM glyoxylic acid monohydrate (Wako Chemicals) and 40 mM HEPES, adjusted to pH 7.0, for 45 min at 4°C. The ganglion was then dried on a slide glass, covered with liquid paraffin, and heated at 100°C for 2.5 min. Serotonin-containing neurons in the heated ganglion exhibited yellow fluorescence (Lent 1982) under a fluorescence microscope, with a mirror unit consisting of an excitation filter (405 ± 5 nm), a dichroic mirror (455 nm), and an emission filter (>455 nm).

Quantitative and statistical analysis

The normalized value of odor-induced mantle shortening in vivo was calculated as follows. The mantle length at three arbitrary time points during spontaneous behavior was measured and averaged (defined as the averaged length). Then the mantle lengths just before and during odorant presentation were measured and normalized to the averaged length. The mantle length itself was defined as described in the preceding text. The number of p-VN spikes in vitro was counted for 30 s preceding or following the onset of the odor stimulus.

For quantitative analysis, the pooled behavioral and electrophysiological data were expressed as the means ± SE. For statistical analysis of the difference between before and during odor application, two-tailed paired t-test was used. An ANOVA was used for comparisons among multiple groups.

RESULTS

Repellent odors selectively elicit mantle contraction via the parietal nerves in vivo

Terrestrial mollusks such as slugs and snails have highly developed olfaction as a sensory modality but not vision or hearing, thus their perception at a distance depends only on their olfactory sense. We observed the behavior of slugs in response to attractive or repellent odorants. Animals approached rat chow, their daily food and an attractive odorant (Fig. 1Ai) but escaped from garlic, an innately repellent odorant (Fig. 1Aii). In escaping from the garlic, animals first showed withdrawal responses and subsequently turned away from the stimulus. Moreover, the withdrawal response was accompanied by contraction of the mantle muscle that covers the head (see Fig. 1B). Conversely, rat chow elicited neither a withdrawal response nor mantle shortening. As shown in Fig. 1C, a quantitative analysis revealed that a significant shortening of the mantle was caused by two repellent odorants, garlic (66.3 ± 7.7% of the control; n = 5) and onion (63.0 ± 11.5% of the control; n = 5), but such shortening was not observed in response to three attractive odorants: rat chow (102.7 ± 4.4% of the control; n = 5), potato (106.9 ± 3.8% of the control; n = 5), and cucumber (96.8 ± 5.3% of the control; n = 5). The mantle shortening in response to the two repellent odors was significant (paired t-test; P < 0.01 in response to the garlic odor, and P < 0.05 in response to the onion odor). These results show that the mantle shortening is one form of defensive behavior and that shortening of the mantle muscle can be used as an index of an aversive response.

We next asked which efferent nerves govern the mantle shortening. Our preliminary observation revealed that a pair of parietal nerves project to the basal region of the mantle and make extensive arborizations to the mantle tip (data not shown; illustrated in Fig. 1B). We then observed the repellent odor-induced changes in the behavior of animals in which the parietal nerves were lesioned bilaterally. The parietal nerve-lesioned animals showed normal locomotive behavior during both spontaneous and food-foraging phases comparable with the sham-operated group. However, lesioned animals showed severe impairment of the mantle shortening in response to the garlic odor (Fig. 1D). Namely, the mantle length in the sham-operated group shortened to 76.7 ± 8.2% of control in response to the garlic odor (paired t-test; P < 0.05; n = 4), whereas garlic odor did not cause a significant reduction in the mantle length in the lesioned group (104.0 ± 5.4%; control; n = 4). These results indicate that a pair of parietal nerves innervate the mantle muscle and mediate the shortening elicited by the repellent garlic odor.

Identification and characterization of a motoneuron (p-VN) innervating the mantle muscle

We identified a motoneuron that projects to the mantle and innervates the mantle muscle. Figure 2A shows the arborization patterns of the newly identified neuron, denoted the p-VN. The soma of this neuron had a diameter of ~80 μm, and its soma and processes were confined to the abdominal ganglion and did not extend into any other ganglia (n = 7). This neuron had axons that descended into the bilateral parietal nerves, the tentacle retractor muscle nerve, and the body wall nerve. We then examined the electrophysiological properties of the p-VN and the parietal nerve in vitro. Two hours after dissection, the parietal nerve spontaneously exhibited discharges with small amplitudes (Fig. 2C, indicated by horizontal arrow). The p-VN did not spontaneously exhibit any spikes (Fig. 2C). When we injected positive DC current into the p-VN, the p-VN exhibited spikes that were recorded at the parietal nerve as much larger discharges (Fig. 2Ci, indicated by horizontal arrowhead). Odor information received at the nose is conveyed to the circumesophageal ganglion via the olfactory nerves (Figs. 1B and 2B). When we electrically stimulated the olfac-
torv nerve, the p-VN exhibited excitatory postsynaptic potentials (EPSPs) and action potentials (Fig. 2Cii, n = 9). The action potentials in the p-VN had an exact one-to-one correspondence with the maximum-amplitude spikes in the left parietal nerve (indicated by the horizontal arrowhead). This exact correspondence was observed in all preparations we examined (n = 9/9). When we applied odor of garlic to the nose, this odor generated action potentials in the p-VN (Fig. 2D, n = 2). Thus the p-VN responds to olfactory input, and the action potentials are propagated into the parietal nerves. In addition, the action potentials could be recorded extracellularly as units of maximum-amplitude spikes produced in the left parietal nerve.

Finally, we examined whether or not the p-VN is the motoneuron that innervates the mantle muscle. In an isolated circumesophageal ganglion with the mantle attached (drawn in Fig. 2B), when a positive DC current was injected into the p-VN to produce action potentials, the action potentials in the p-VN produced one-for-one EJPs at the mantle muscle (Fig. 2E, n = 3). This connection between the p-VN and the mantle muscle is monosynaptic because the synaptic latency between the peak of p-VN spikes and the EJP is constant (Fig. 2F, n = 3) and short (15.9 ± 3.3 ms; n = 3). The synaptic latency was measured as the period from the peak of p-VN spikes to the onset of the EJP. These characteristics are consistent with that of other monosynaptic connections in Limax. [For example, the synaptic latency of monosynaptic connection between the olfactory nerve and nonbursting neuron in the PC is ~25 ms. (see Inoue et al. 2000)]. These results indicate that the p-VN is one of the motoneurons that project to the mantle and innervate the mantle muscle. We could not test whether or not discharges in the p-VN actually elicit the mantle shortening directly by tension-meters, because of the technical difficulty: Limax marginatus is too small to record it.

Repellent odors selectively elicit p-VN discharges in vitro

We looked at activity changes in the p-VN when various attractive and repellent odors were applied to the nose of the isolated whole ganglia (Fig. 2B). Action potentials in the p-VN were monitored by extracellular recording of the left parietal nerve. The isolated slug brains were divided into two groups. In the first group (group A), we tested two odorants, rat chow as an attractive odor and garlic as a repellent one (Fig. 3Ai). In the second group (group B), we used three odorants, potato and cucumber as attractants and onion as a repellent (Fig. 3Aii). As shown in Fig. 3A, the attractive odors of rat chow, potato, and cucumber hardly changed the activities of the left parietal nerve or the p-VN. On the other hand, application of the repellent odors of garlic and onion increased the unit activity of the left parietal nerve that corresponded to spiking of the p-VN (indicated by the horizontal arrowhead).

Quantitative analysis of the spiking activities of the p-VN during the pre-odor or odor-stimulus period is shown in Fig. 3B. Spiking rates of the p-VN were almost identical during the pre-odor period with all odors, and the differences were not statistically significant (paired t-test; P = 0.62 in group A; ANOVA, P = 0.51 in group B). In group A (n = 7), application of rat chow odor caused little change in the spiking rate of the p-VN (0.43 ± 0.32 counts/30 s for pre-odor vs. 1.00 ± 0.53 counts/30 s for odor stimulus), whereas garlic odor dramati-
odor was statistically significant (paired t-test; \( P < 0.01 \) versus pre-odor). In group \( B \) (\( n = 7 \)), the potato odor elicited no change in the spiking rate of the p-VN (0.57 ± 0.32 counts/30 s for pre-odor vs. 0.71 ± 0.45 counts/30 s for odor stimulus), and the cucumber odor produced only a slight increase (0.43 ± 0.22 counts/30 s for pre-odor vs. 2.86 ± 0.98 counts/30 s for odor stimulus), whereas the onion odor markedly increased the number of spikes of the p-VN (0.57 ± 0.32 counts/30 s for pre-odor vs. 14.86 ± 3.85 counts/30 s for odor stimulus). The increase in spiking rate induced by the onion odor was statistically significant (paired t-test; \( P < 0.01 \) vs. pre-odor). Spiking activity induced by the cucumber odor also showed a statistically significant increase (paired t-test; \( P = 0.037 \) vs. pre-odor), but the odor of cucumber did not increase the number of spikes as much as the two repellent odors garlic and onion. These results demonstrate that repellent odors selectively increase the number of p-VN spikes, i.e., that odor-induced behavioral changes in vivo can be monitored in vitro, using the spiking activity in the parietal nerves and the p-VN.

Identification and characterization of a serotonergic neuron (p-CSC) projecting to some olfactory processing regions

In \textit{Limax} and related species, odor information received at the nose is first transmitted to the TG and subsequently transmitted to the PC (Chase 1986). Both the TG (Inokuma et al. 2002) and the PC (Gelperin and Tank 1990) exhibit network oscillations, which are modulated by odor input to the nose. Neurons that project to these regions, the TG or PC, are prime neuronal candidates for regulating odor-induced discharges in the p-VN, i.e., odor-induced aversive behavior. We next looked for such a neuron.

Figure 4 shows an identifiable serotonergic neuron. The neuron we identified exhibited yellow fluorescence when we applied glyoxylic-acid staining to the cerebral ganglion of \textit{Limax} (Fig. 4B; indicated by arrowhead; \( n = 4 \)). Such yellow fluorescence means that the identified neuron is a serotonin-containing neuron (Lent 1982). The soma of this identifiable serotonergic neuron is \( \sim 30–40 \mu \text{m} \) in diameter and is located at the posterior side of the cerebral ganglion. Therefore we named the neuron the p-CSC. Intracellular staining of the p-CSC, by intracellular biocytin filling, revealed that the p-CSC extends the neurites extensively to the PC and also has a thick axon to the olfactory nerve (Fig. 4C; \( n = 3 \)). We could trace this axon of the p-CSC to the TG beyond the olfactory nerve but could not observe the arborization inside the TG. However, as described in Fig. 5, we could electrophysiologically observe the serotonergic projection to the TG (see following text). These results indicate that the p-CSC is an identifiable serotonergic neuron, which projects to the PC and probably to the TG.

Excitation in the p-CSC releases serotonin to the tentacle ganglion (TG)

Is the TG actually an output region of the p-CSC? We simultaneously recorded the p-CSC (intracellular recording) and LFP in the TG (Fig. 5) to electrophysiologically investigate the projection of the p-CSC to the TG. Discharges in the p-CSC, elicited by injection of a positive DC current, induced \( \sim 1 \)-Hz LFP oscillation in the TG (Fig. 5Bi). The power spectrum clearly indicates a remarkable increase in oscillatory power with a range of \( \sim 1 \) Hz (Fig. 5Bi). The averaged oscillatory frequency was 1.03 ± 0.04 Hz (\( n = 3 \)). If discharges in the p-CSC directly induce the 1-Hz network oscillation, direct application of serotonin to the TG should also generate such a network oscillation because the p-CSC is serotonergic. We found that local application of serotonin to the TG with a fine puffing pipette also elicited a LFP oscillation with the same frequency range (Fig. 5C; \( n = 3 \); the averaged frequency is 0.93 ± 0.08 Hz). These results indicate that discharges in the p-CSC release serotonin to the TG and induce a network oscillation in the TG.

Serotonin in the TG inhibits repellent odor-induced aversive behavior

Finally, we examined the effect of serotonin in the TG on odor-induced p-VN discharges (our in vitro index of aversive behavioral response) as shown in Fig. 6. In this experiment, we used a pure organic chemical (amyl acetate; AA) diluted with the physiological saline as the odorant. The reason is described in Odor and electrical stimulation.

AA is a behaviorally aversive odorant. Application of AA, from a fine puffing pipette (\( \sim 5 \mu \text{m} \) in tip diameter) containing 0.1% AA, elicited p-VN discharges (Fig. 6B; \( n = 4 \); 14.25 ± 2.59 counts/30 s for AA application). The number of p-VN spikes induced by the AA was almost the same as those induced by two aversive odors shown in Fig. 3, garlic (14.29 ± 3.36 counts/30 s) and onion (14.86 ± 3.85 counts/30 s). This suggests that, at the concentration we used, AA is a physiologically aversive odor like garlic and onion.

We next examined the effect of serotonin to the TG on discharges in the p-VN. Selective application of serotonin to the TG (see METHODS for details) induced the network oscilla-
DISCUSSION

There are three new findings in the present paper. First, we found that shortening of the mantle muscle is one of the withdrawal responses induced by aversive odors in Limax (Fig. 1). Second, we identified p-VN and parietal nerve discharges as an in vitro index of odor-induced aversive behavior in Limax (Figs. 2 and 3). Third, we identified the p-CSC, a serotonergic neuron that regulates the odor-induced aversive behavior (Figs. 4–6). We now discuss these findings in greater detail.

Discharges in the p-VN and parietal nerve: an in vitro index of odor-induced aversive behavior

In general, in vitro systems using isolated molluscan brain is very useful for investigating the neural network. Isolated molluscan brains with sensory organs and muscle are still alive under physiological saline. In addition, most areas of the molluscan nervous system (except for the PC) consist of a few big and identifiable neurons, making it easier to identify the neural connections. There are many good studies on PC network dynamics in Limax (Gelperin and Tank 1990; Kimura et al. 1998b; Kleinfeld et al. 1994), but there are no studies on in vitro motor output that can explain odor-guided behavior in Limax. Identification of the motor output is very important for elucidating the output of PC network dynamics. In the present study, we first found that mantle shortening is one of the aversive behavioral responses in Limax (Fig. 1, A–C). This mantle shortening is inhibited by bilateral lesioning of the parietal nerves (Fig. 1D). Figure 1 shows that aversive odor-induced mantle shortening is mediated by the parietal nerves. Odor application to the nose in isolated brains revealed that only aversive odors (garlic and onion) elicited discharges in the parietal nerve (Fig. 3). As shown in Fig. 3A, aversive odors activated some efferent neurons with different amplitudes, whereas attractive odors did not induce any change in these neurons. The results in Fig. 3 indicate that the parietal nerve discharged selectively in response to aversive odors in vitro; this means there is behavioral correspondence between in vivo (Fig. 1) and in vitro (Fig. 3). Furthermore, we identified a motoneuron, the p-VN and found that spiking in the p-VN corresponded to the unit of highest amplitude in the parietal

FIG. 5. The p-CSC releases serotonin to the TG and induces a 1-Hz network oscillation in the TG. A: schematic diagram of the experimental design in Fig. 5. B: network oscillation in the TG induced by p-CSC stimulation. Bi: representative traces. The p-CSC was intracellularly recorded by a conventional sharp electrode, and discharges in the p-CSC were elicited by a DC-positive current injection. Discharges in the p-CSC induced a 1-Hz LFP oscillation in the TG. Bii: the power spectrum of the TG oscillation before (…) or during (---) p-CSC stimulation. Scale bars are 4 s, and 5 μV (TG LFP) or 20 mV (p-CSC). C: a network oscillation in the TG induced by direct application of serotonin to the TG. Ci: representative traces. Serotonin was puffed from a fine glass pipette (≈5 μm in the tip diameter). Serotonin, puffed to the TG, also induced the LFP oscillation that had the same frequency range as the LFP oscillation induced by p-CSC stimulation. Cii: the power spectrum of the TG oscillation before (…) and during (---) serotonin application to the TG. Scale bars are 5 s and 10 μV.
nerve (Fig. 2). This neuronal identification of the p-VN is a great advantage for elucidation of the exact neural connection in Limax, although other efferent neurons with axons in the parietal nerve might also be important for mantle shortening.

Our results clearly indicate that the parietal nerves mediate the shortening of the mantle muscle because lesioning the parietal nerve might also be important for mantle shortening in vivo inhibited the mantle shortening in response to an aversive garlic odor (Fig. 1D), and discharges in the parietal nerves were induced selectively in response to aversive odors (Fig. 3). We have also shown that the p-VN is a motoneuron that innervates the mantle muscle (Fig. 2) and selectively exhibits the discharges in response to aversive odors (Fig. 3). Action potentials in the p-VN generate one-to-one EJPs at the mantle muscle (Fig. 2E), and the latencies between the peak of p-VN spikes and onset of EJPs are constant (Fig. 2F). These results indicate that the p-VN makes a monosynaptic connection with the mantle muscle and innervates the mantle muscle.

The advantage of having an in vitro index of odor-induced aversive behavior (p-VN and parietal nerve discharges) is that we can study the relationship between brain activities and behavior in vitro. In experiments in vivo, we observe the animal’s behavior so that we can judge whether animals learn tasks or not. However, in experiments in vitro, there are only isolated brains and no bodies (i.e., no behavior), and thus we usually cannot judge learning of animals under in vitro experimental conditions. Therefore, the in vitro index of odor-induced aversive behavior we found is a great advantage to investigate the relationship between brain activities and learning and memory in vitro. Our preliminary data demonstrate that in vitro nose-brain-motor output preparations have a learning ability for odors; they could discriminate the quality of different odors in vitro (Inoue et al. 1999).

The p-CSC: an identifiable serotonergic neuron regulating the odor-induced aversive behavior

Our next task was to identify the neuronal component that regulates the odor-induced discharges in the p-VN. We identified the p-CSC, an identifiable serotonergic neuron in the cerebral ganglion of Limax (Fig. 4). Discharges in the p-CSC release serotonin into the TG (Fig. 5), and serotonin in the TG suppresses aversive odor-induced discharges in the p-VN (Fig. 6). This evidence suggests that the p-CSC regulates the odor-induced discharges in the p-VN; i.e., discharges in the p-CSC might inhibit aversive behavior in response to aversive odors.

How does serotonin in the TG suppress the odor-induced discharges in the p-VN? Anterograde labeling from the olfactory nerve revealed that TG neurons have three nerve tracts in

![image]
the olfactory nerve (data not shown). One tract projects to the PC, a second tract projects to the AG, and the third tract projects to the CG outside the PC. The p-VN is located in the AG, and electrical stimulation in the olfactory nerve elicited EPSPs in the p-VN with about a 10-msec latency (see Fig. 2Cii). This latency is shorter than that in the olfactory nerve → PC neuron synapse (~25 ms) (see Inoue et al. 2000). These morphological and electrophysiological findings indicate that the TG → p-VN pathway is a direct and excitatory one and is not mediated by the PC. We have no data suggesting how serotonin in the TG suppresses the odor-induced discharges in the p-VN. One possibility is that this suppressive effect is achieved when the serotonin in the TG hyperpolarizes the TG neurons that project directly to the AG and the p-VN. Previous morphological findings by retrograde labeling from the olfactory nerve to the TG have revealed that there are several types of neurons in the TG (Chase and Kamal 1983; Ito et al. 2000). Our hypothesis could be experimentally tested through intra-cellular recording combined with morphological identification of each TG neuron.

Although our data in Figs. 5 and 6 suggest that the p-CSC inhibits odor-induced discharges in the p-VN, we could not determine it by simultaneous recording of the p-CSC and odor-induced discharges in the parietal nerve (p-VN). This is due to an inherent technical difficulty: a very important nerve tract for odor-induced discharges in the p-VN (TG → p-VN direct pathway described in the preceding text) runs just on the soma of the p-CSC. As a result, penetration of the p-CSC by a sharp electrode inevitably injures the nerve tract. Actually, we encountered a lot of discharges in the p-VN when we were penetrating the p-CSC with a sharp electrode probably due to injury of the nerve tract by the sharp electrode.

Another question about the p-CSC is the mechanism how p-CSC stimulation evokes large LFP oscillatory activity in the TG (Fig. 5B). Such a large oscillatory activity was also evoked by a direct application of serotonin to the TG (Fig. 5C) (Inoue et al. 2002). Thus serotonin evokes the large LFP oscillatory activity. Our preliminary result using the technique of perforated patch-clamp recording demonstrated that membrane potentials in the TG neuron exhibit large amplitude of slow oscillation and bursts by application of serotonin (unpublished observations). This slow oscillation of the membrane potentials was synchronized with the LFP oscillation in the TG. Thus it is suggested that the slow oscillation in the TG neurons is involved in large LFP oscillatory activity in the TG. Further investigation of effect of serotonin on the TG neurons will clarify the mechanism of serotonin-induced large LFP oscillatory activity in the TG as well as the mechanism how serotonin in the TG suppresses odor-induced spikes in p-VN.

Interaction between the PC and the identified pathway of nose—motor output

In Limax and related species, the PC is the center of olfactory information processing (Chase 1985; Kimura et al. 1998a). PC neurons exhibit ongoing synchronized oscillation of their membrane potentials (Gelperin and Tank 1990). This type of network oscillation is also observed in mammalian (Adrian 1950; Freeman 1978) and insect (Laurent and Naraghi 1994) olfactory centers. To elucidate the physiological role of such network oscillation on the decoding to behavior, we need to identify a neural pathway from the oscillatory olfactory center to behavioral output. To date, some follower neurons of the PC have been identified (Chase and Tollozczko 1989; Gelperin and Flores 1997; Shimozono et al. 2001). These neurons project their neurites to the PC and exhibit the neuronal activity that is phase-locked with the network oscillation in the PC. However, there is no report that these neurons actually regulate behavioral outputs. Thus it is an interesting question whether or not the PC and the p-CSC interact, because the p-CSC is a behavior-regulatory neuron that suppresses odor-induced p-VN discharges, an in vitro index of odor-induced aversive behavior (Figs. 4–6).

Our preliminary data demonstrated that kainate had an excitatory effect on PC neurons, inducing spiking in the PC neurons. When we applied kainate selectively to the PC, the kainate excited the p-CSC and generated spikes in the p-CSC. Thus these preliminary data suggest that there is an excitatory connection from the PC to the p-CSC, although we do not know whether the connection is monosynaptic or polysynaptic. On the other hand, discharges in the p-CSC modulated the LFP oscillation in the PC (data not shown). Such modulation in the PC could be induced by direct application of serotonin to the PC (Gelperin et al. 1993). This suggests that there is a serotonergic connection from the p-CSC to the PC. These preliminary data suggest that the PC neurons have a mutual connection with the p-CSC. However, the interaction between the PC and the p-CSC was complicated and, therefore we will report these issues in another paper. It is no doubt that the clarification of the interaction between the PC and p-CSC will enable us to understand the interaction between the PC and the odor-induced motor pathway identified in this paper.

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Disclosures

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