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Contributions of Superior and Inferior Tentacles to Learned Food-Avoidance Behavior in *Limax marginatus*

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ABSTRACT—We investigated learned food-avoidance behavior of conditioned slugs in detail, and examined the effect of removing the inferior or superior tentacles (ITs and STs) on the avoidance behavior. The conditioning procedure significantly lowered the preference level of slugs to the conditioned odor, and the decrease of the preference was maintained for at least 48 hr. Video analysis experiments showed that change in the crawling direction during an aversion response occurred consistently accompanied by an average of 1.2 head swings. The first head swing was usually observed within 1 cm from the conditioned odor. Removal of the ITs after conditioning reversed the trend towards a lowered preference level. On the other hand, removal of the STs did not change the level. These results suggest that the IT inputs are needed to induce conditioned behavior. The contribution of STs to the conditioning behavior is indicated by the significant increase in the average number of head swings (3.6 swings) in a trial after their removal. In some trials, the slugs lacking STs avoided the odor source after crossing it. These observations indicate that removal of STs decreases the orientation ability of conditioned slugs.

INTRODUCTION

Many species of vertebrates and invertebrates have a pair of accessory olfactory organs as well as the main olfactory organs to perceive chemical stimuli related to sexual or social behavior (Halpern, 1987; Wysocki and Meredith, 1987). For example, the vomeronasal organ of vertebrates has been shown to be important in reproductive behavior of both sexes (e.g. Fleming *et al*., 1979; Marques 1979; Wysocki 1979; Rajendren *et al*., 1990). Terrestrial molluscs have a pair of shorter tentacles named the inferior tentacles (ITs) in addition to the longer ones (superior tentacles; STs). The function of ITs has been reported to be related to mucus trace behavior (Gelperin, 1974).

On the other hand, it has also been reported that vomeronasal organs are used for food searching (e.g. Jiang *et al*., 1990). Some physiological experiments have revealed that vomeronasal organs respond to various general odorants (Hatanaka *et al*., 1988; Shoji and Kurihara, 1991). In molluscs, it has also been shown that the ITs can detect some foods from a distance of a few millimeters (Gelperin, 1974; Cook, 1985). These findings indicate that accessory olfactory organs have a role in sensing general odors such as those from food. Therefore, we consider that if olfactory mechanisms are to be understood, we need to clarify how accessory organs, as well as the main olfactory organs, sense general odors.

In terrestrial slugs, afferent fibers arising from the IT tip, in common with those arising from the ST tip (Kimura *et al.,* 1998a), innervate the terminal mass of the procerebral (PC) lobe of the cerebral ganglion. The neuronal network of the PC lobe in the slug *Limax* exhibits spontaneous oscillatory activity (Gelperin and Tank, 1990; Kawahara *et al*., 1997) whose frequency changes when the ITs respond to odors (Kimura *et al*., 1998c). The types of changes in this spontaneous activity reflect how the slug has been conditioned (Kimura *et al*., 1998b c). These findings suggest that inputs from the ITs are important for the process of olfactory recognition necessary to learned odor aversion, whereas it has been reported that inputs from ITs in *Helix* are not required in searching for foods that the slug has learned to be appetizing (Friedrich and Teyke, 1998).

In the present study, we addressed the question of which input pathways are involved in learned avoidance behavior in *Limax*, and how selective tentacle cutting affects learned food avoidance behavior.

MATERIALS AND METHODS

Animals

Limax marginatus individuals were maintained in our laboratory on frog chow (Oriental Yeast Co. Ltd., Tokyo) with a light/dark cycle of 14/10 hr at 19°C. Prior to the start of the experiments, each slug was separated in a plastic chamber (113×105×28 mm) and kept for 3 or more days without food to increase its sensitivity to food odors. The weight of each slug used in the experiments was 1.5–2.0 g before starvation.

Conditioning training

Each of the slugs was transferred with tweezers onto a glass

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plate (30 × 45 cm). Though the animals were initially disoriented and sometimes spontaneously changed their direction of crawling just after transfer, they always became acclimated within about 5 min and began to crawl forward. A line of carrot juice was then applied by Pasteur pipette to the plate at right angles to the body axis and 0.5–1.0 cm far from the head. About 10 sec later, just before each slug touched the odor source, 100 µl of quinidine sulfate solution saturated in slug saline was dropped onto the skin near the lip as an unconditioned stimulus. After a further 10 sec, the quinidine sulfate was washed off with slug saline (NaCl 70.0 mM, KCl 2.0 mM, $CaCl_2$ 4.9 mM, $MgCl_2$ 4.6 mM, glucose 5.0 mM, HEPES 10 mM; pH 7.6) and the animal was returned to its chamber. In these experiments, the conditioning procedure was applied twice for each slug with an interval of more than 2 hr.

Control training

The conditioning odor of carrot was presented to slugs as described above. In unpaired control groups, each animal was removed gently from the glass plate and transferred to a holding chamber just before it touched the odor source. Fifteen minutes later, the slug was again placed on the glass plate, and quinidine sulfate solution applied as described above.

Tentacle removal

After slugs had been anesthetized by cooling (4°C, 30 min), the ITs or STs were cut off bilaterally with scissors. Tentacles were always removed more than 6 hr after the last control odor or conditioning training. This delay reduced the influence of cutting or cooling on odor memory.

Test

About 6 hr after the last conditioning or tentacle cutting, the response of the animals to food odor was examined. Each animal was placed on a glass plate as described above and left to acclimate. After it had begun straight crawling, a line of odor source was applied 5–10 cm ahead of the slug in the same manner as that for conditioning. If slugs did not crawl, we sometimes gently touched the skin surface with tweezers to encourage them to do so. The observation period for each test was 5 min. Personnel who did not know the history of each animal completed these tests. When the preference level was compared, Fisher's exact probability test was used.

When the effect of tentacle removal was examined in control and conditioned groups, in this experiment, each slug was trained twice, and its response to carrot odor was tested before IT removal. Six hours after tentacle removal, the same animals were tested again.

Quantitative analysis of avoidance behavior

The behavior near the odor source was also recorded by a video camera (SANYO, VM-ES805). The videotape was played back on a high-resolution TV monitor, and the head position and head angle were measured at each 1-sec interval from the paused view. The head position was defined by the distance from a middle point between the basal parts of the right and left superior tentacles to the nearest edge of the odor source. The head angle was measured as the angle between the perpendicular axis of the line of odor source and the head axis (defined as the longitudinal stripe on the head of the slug). In many cases, the conditioned slugs showed rapid head swinging near the conditioned odor source, and the head swing number in a trial was also analyzed. A head swing was defined as any change in the head angle greater than $\pi/3$ in 5 sec. The mean number of head swings per trial or the mean value of the head position, where the first head swing was observed, in each group was compared using the Mann-Whitney U test.

RESULTS

Changes in odor preference

Kimura *et al*. (1998c) have reported previously for *Limax marginatus* that the conditioning method employed in the present study selectively decreases their preference for the conditioned odor. Prior to the tentacle removal experiments, we examined the change in conditioned odor preference for 48 hr. In this study, the preference of each group for the conditioned odor (carrot odor was used in all experiments) was defined as the percentage of slugs which succeeded in eat-

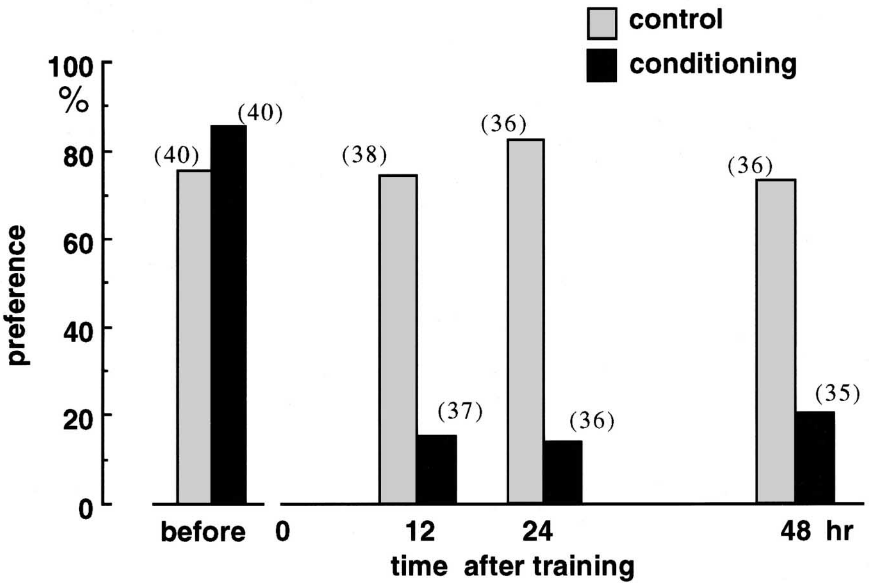

Fig. 1. Effects of conditioning (gray column) and control training (black column) on the preference to conditioned odor for 48 hr. Each column shows the preference of one slug group, which detected the odor before conditioning (before), and 12 hr, 24 hr or 48 hr after conditioning. The number above each column shows the number of slugs used in each session.

ing the presented odor source in a test. Figure 1 shows the effects of control and conditioning training on preference. Before training, slugs in both groups showed a high level of preference for the carrot odor (p>0.05 Fisher's exact probability test; Fig. 1 before training). After training, a decrease in preference level occurred only in the conditioned group (Fig. 1, 12 hr). The difference in preference level between the control and the conditioned group was maintained for at least 48 hr with 3 test sessions (p<0.01 in all cases by Fisher's exact probability test; Fig 1, 12, 24 and 48 hr). Based on this result, we performed the first and second test sessions within 48 hr after training.

Learned avoidance behavior of intact slugs

A typical example of change in head position and head angle during learned avoidance behavior is shown in Fig. 2. The head position plot shows that, at first, the conditioned slug gradually approached the odor source, but when it was within 1 cm of the odor, it began to avoid it (Fig. 2A). The head angle plot indicated that rapid head turning occurred (at the start of avoidance (white arrow in Fig. 2B). At this point, the slug stopped crawling and lifted its head (black arrow in Fig. 2B), and then head swinging was observed (white arrow in Fig. 2B). It has been reported that the turning behavior of molluscs is used as a mechanism of orientation to an odor gradient (Friedrich and Teyke, 1998). Therefore, we measured the number of head swings needed to determine the avoidance direction in each test as a parameter of the slug's ability to orientate relative to an odor gradient. In intact slugs, the number of head swings observed in one avoidance behavior was

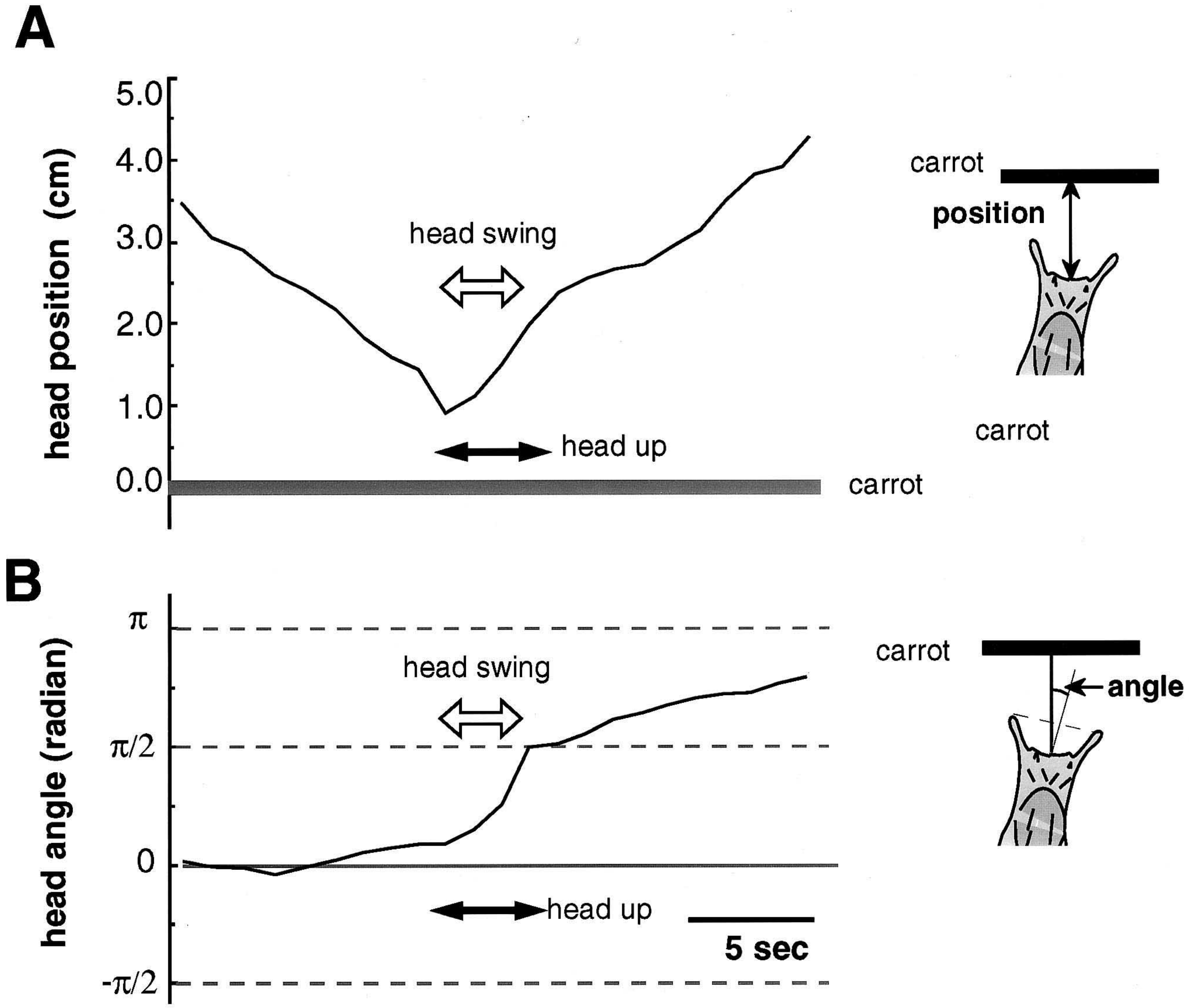

Fig. 2. Change in head position (A) and head angle (B) during avoidance behavior in an intact conditioned slug. Head position (distance from odor source) and head angle (angle between the axis perpendicular to the odor source and the longitudinal axis of the head) was obtained from each 1 sec frame of the videotaped view. When the slug approached to within 1 cm of the odor source (A), it changed its crawling direction accompanied by head raising (black arrow in B) and head swinging (white arrow in B). The period of head raising was determined from the recorded view.

1.2±0.5 (mean±SD; 22 tests in which the avoidance occurred in 15 conditioned slugs). Furthermore, we also measured the head position at which the first head swing was observed as a parameter of the slug's sensitivity to the carrot odor. In intact slugs, the first head swing was observed mainly after the animal came within 1 cm from the odor source (Fig. 3).

Effect of IT removal on the preference level

The effect of IT removal was examined in the control and the conditioned groups. Before IT removal, the control slugs showed a significantly higher level of preference for carrot odor (Fig. 4A before removal) than the conditioned group (Fig. 4B, before removal) ($p<0.01$, Fisher's exact probability test). However, IT removal induced a significant increase of carrot odor preference in the conditioned slugs (Fig. 4B), and there

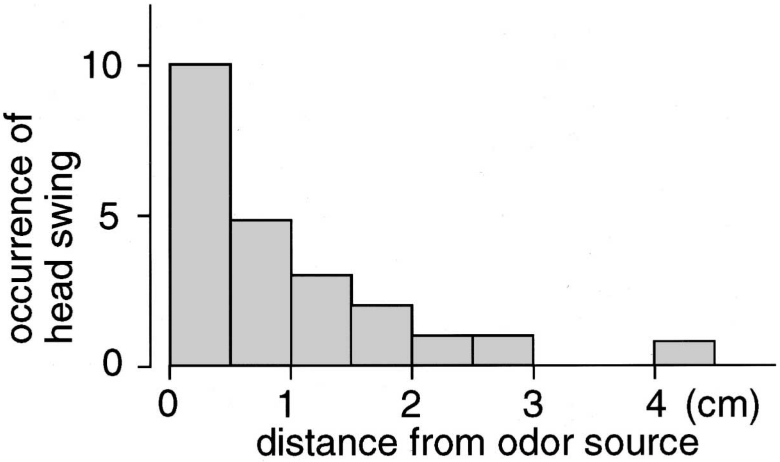

Fig. 3. Distribution of the starting points of head swinging in an intact group of conditioned slugs. In this experiment, 15 animals were used and the response was testedtwice in each. The histogram shows the number of first head swings in each 5-mm distance from the odor source.

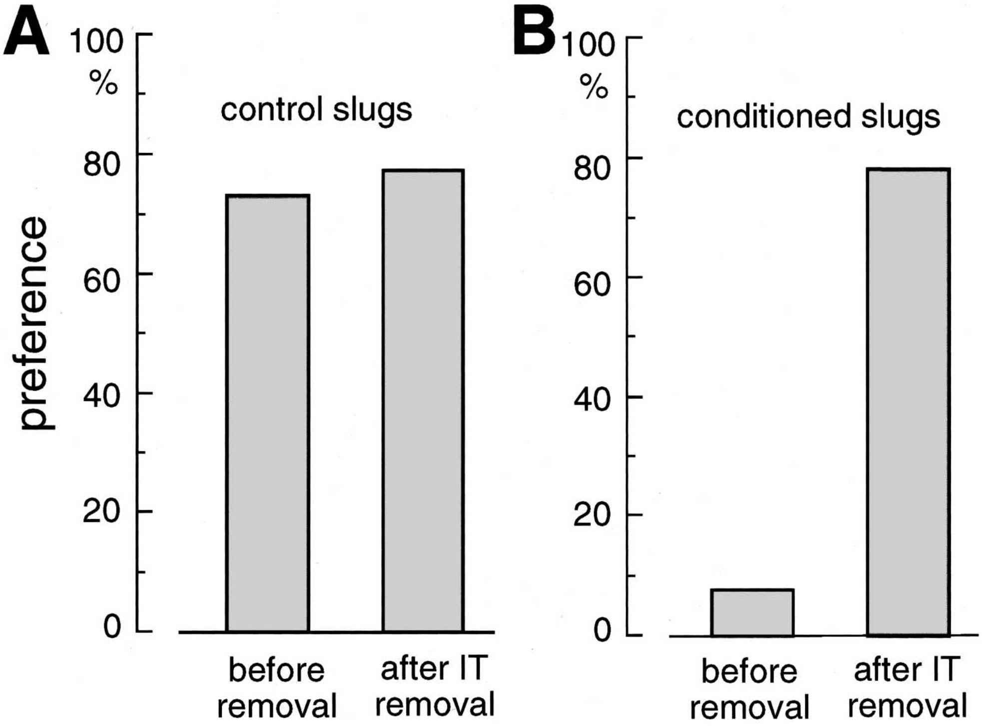

Fig. 4. Effect of IT removal on preference in control (A) and conditioned slugs (B). Each of the data represent the results of 40 tests using 40 slugs. In this experiment, the preference of each group was measured before (before removal) and after (after IT removal) tentacle removal. No significant difference was detected between the two groups after IT removal (after IT removal in A and B), although there is a significant difference between them before removal (before removal in A and B).

was no significant difference in odor preference between the control and the conditioned slugs (Fig 4A, B, after IT removal) ($p>0.05$, Fisher's exact probability test). These results suggest that inputs from the ITs are important for inducing the learned avoidance behavior.

Effect of ST removal on the preference

Behavioral changes before and after ST removal were examined in the control (Fig. 5A) and conditioned (Fig. 5B) groups. In the control group, there was no significant difference in the odor preference before and after ST removal ($P>0.05$, Fisher's exact probability test). In addition, ST removal in the conditioned slugs also induced no significant change in preference ($p>0.05$ in both cases, Fisher's exact probability test), and the difference in preference level between the control and conditioned slugs was maintained after ST removal ($P<0.05$, Fisher's exact probability test).

The head position at which the head swing was observed

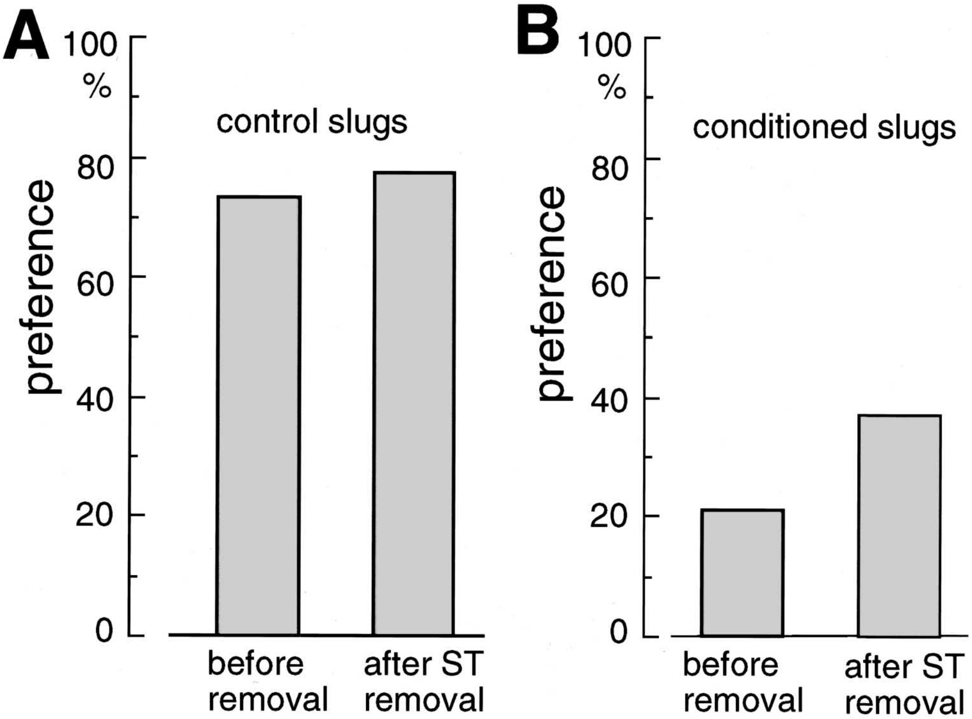

Fig. 5. Effect of ST removal on preference in control (A) and conditioned slugs (B). Each of the data represent the results of 40 tests using 40 slugs. In this experiment, the preference of each group was measured before (before removal) and after (after ST removal) tentacle removal. A significant difference was detected between the two groups ($P<0.05$, Fisher's exact probability test), although the STs were removed (after ST removal in A and B).

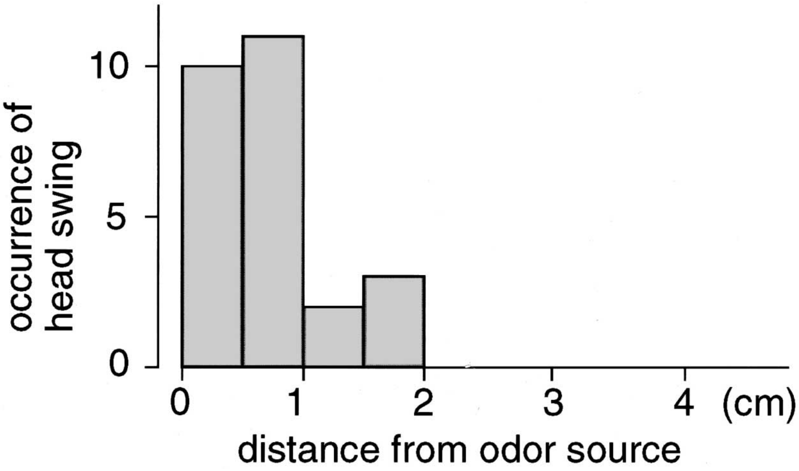

Fig. 6. Distribution of the starting points of head swinging in a group of conditioned slugs lacking STs. In this experiment, 15 animals were used and the response was tested twice in each. The histogram shows the number of first head swings in each 5-mm distance from the odor source.

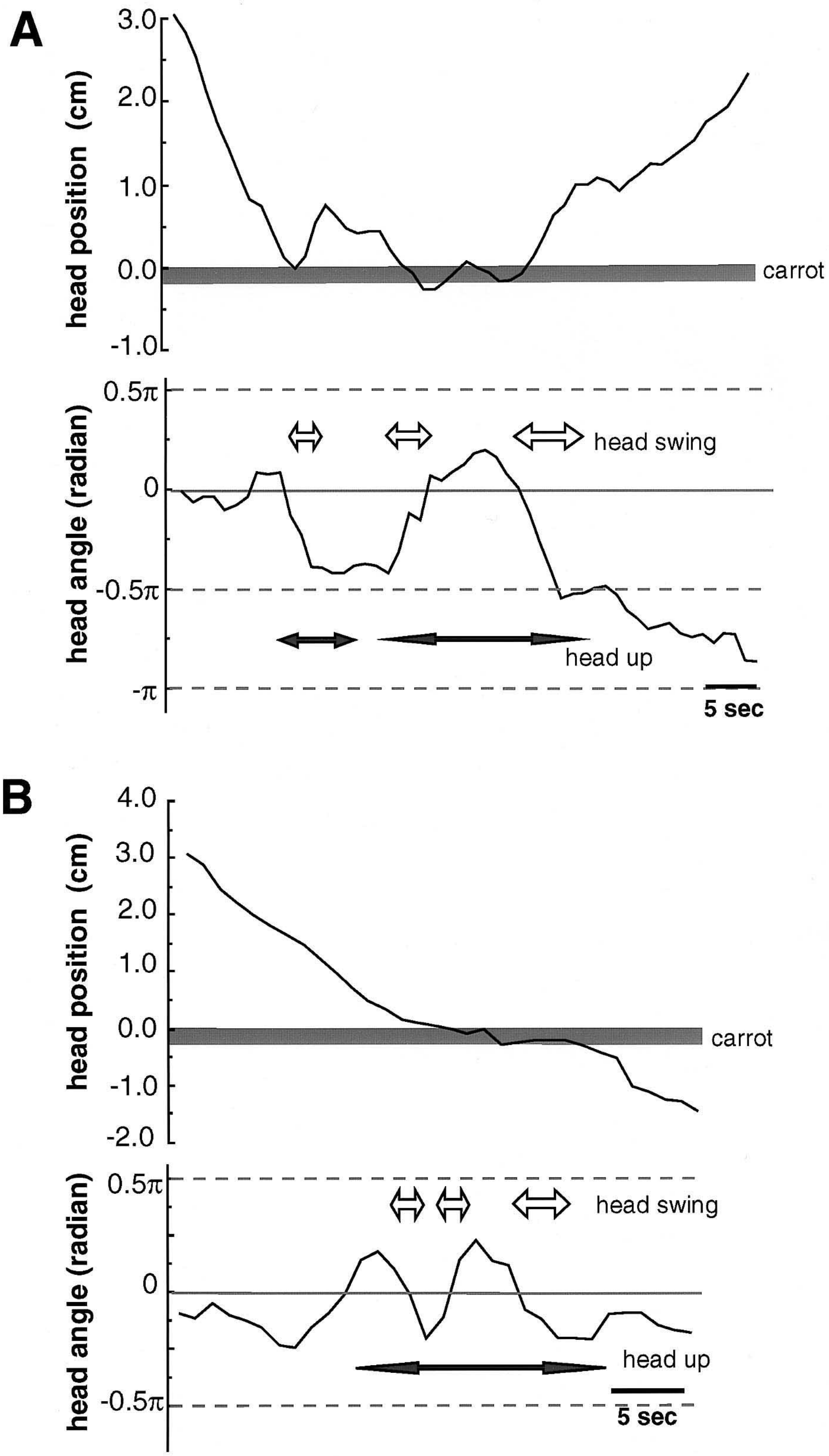

Fig. 7. Two typical examples (A and B) showing changes in head position (upper graphs in A and B) and head angle during avoidance behavior (lower graphs in A and B) in two conditioned slugs lacking STs. The head position (distance from odor source) and head angle (angle between the axis perpendicular to the odor source and the longitudinal axis of the head) were obtained from each 1-sec frame of the videotaped view. A) A conditioned slug lacking STs showed head raising twice (black horizontal bars) and head swinging three times (gray horizontal bars) after closing the presented odor source, and changed its crawling direction to avoid the odor source. B) Another conditioned slug lacking STs showed head raising once (black horizontal bar) and head swinging three times (gray horizontal bars) after closing the presented odor source, and then it passed over and away from the odor source.

in each trial was measured in the slugs lacking STs in comparison with that in the intact slugs. The distribution of the head position showed that the head swing often occurred within 1 cm of the odor (Fig. 6). There was no significant difference in head position between intact slugs and slugs lacking STs ($p>0.05$, Mann- Whitney U-test). These results suggest that the avoidance behavior shown by slugs lacking STs is very similar, in terms of both odor preference and sensitivity, to that of intact slugs.

Effect of ST removal on avoidance behavior

After ST removal, we found two types of avoidance behavior in the slugs. Changes in head position and head angle in one type of behavior are shown in Fig. 7A. First, the slug crawled towards the line of carrot extract presented as the conditioning odor source (Fig. 7A, head position). Then, after approaching the extract, the slug lifted its head and swung it three times to avoid the odor (Fig. 7A, head angle). In the other type of avoidance behavior (Fig. 7B), although head raising and head swinging (Fig. 7B, head angle) were observed after approaching the odor source (Fig. 7B, head position), the slug proceeded to cross the odor region without its head coming into direct contact with the surface (Fig. 7B, head position). The latter type of behavior is also categorized as a form of avoidance, because it enables slugs to avoid the odor source (Fig. 7B, head position). This type of avoidance behavior, which involves avoidance without a change in the direction of crawling, was sometimes observed in slugs lacking STs (8 of 24 avoidances observed by video analysis), but hardly detected in intact slugs (0 of 22 avoidances observed by video analysis). These observations suggest that removal of the STs would induce a significant change in orientation ability.

To examine this hypothesis, we measured the number of head swings needed to determine the avoidance direction in each trial. In slugs lacking STs, the mean number of head swings during one avoidance was 3.1 ± 1.6 (mean±SD; 24 trials in which the avoidance occurred in 15 conditioned slugs), which was significantly greater than that in intact conditioned slugs ($p<0.01$, Mann-Whitney U-test). This result also suggests that the orientation ability of slugs lacking STs is lower than that of intact slugs.

DISCUSSION

IT removal increased the level of preference for a learned aversive odor in slugs. On the other hand, ST removal did not influence preference for the conditioned odor in conditioned slugs. These results indicate that the influence of tentacle cutting on the preference depends on the tentacles that are removed. Therefore, it is strongly suggested that the effect of IT removal is not due to a general injury effect, although there is no evidence to show that the damage to slugs caused by the cutting of these tentacles is similar. The results also suggest strongly that IT inputs are important for inducing conditioned behavior. We measured the distance of the head from the odor source (head position) to study the role of the ITs in odor detection, since it has been reported that IT input is used for food searching within a few millimeters of an odor source (Gelperin, 1974). The first head swing in each trial was usually observed when the slug was 1 cm from the odor source, and this tendency was maintained in the slugs that possessed only ITs. This suggested that intact slugs also used the IT input to determine their response to the odor. Kimura *et al.* (1998a, b) reported that IT inputs modulated the oscillatory activity of the ipsilateral PC lobe, and that the type of modulation depended on prior experience. Physiological data also support the importance of IT inputs in inducing learned aversive behavior.

Chase and Croll (1981), in a study of *Achatina*, used a Y-shaped olfactometer to show that exposure of STs to any aversive odor did not reliably elicit head turning away from the stimulated side, although attractive odors induced positive orientation. The present experiments revealed that conditioned slugs lacking ITs rarely showed aversive behavior. The findings in *Achatina*, and our tentacle removal experiments, suggest that inputs from the STs are not directly involved in induction of the odor aversion behavior. On the other hand, the analysis of learned avoidance behavior indicated that ST removal induced an increase in the number of head swings per trial. It has been reported that turning behavior has an important role in detection of an odor gradient in molluscs (Friedrich and Teyke, 1998). Based on this fact, it is considered that a decrease of orientation ability induces an increase in the number of turns needed for orientation. In this study we found that the number of head swings needed for negative orientation (Fig. 2) increased after ST removal (Fig. 7). As the head swing is a form of turning behavior, the present results suggest that ST removal decreases orientation ability. It has also been reported that in a snail, olfactory inputs from the STs are related to two important types of food-searching behavior, anemotaxis (orientation toward a wind-borne odor) and tropotaxis (orientation to an odor gradient) (Chase, 1981, 1986). Thus, it is considered that inputs from the STs are important for detecting an odor gradient in both food aversive and attractive behavior.

It is concluded from this study that IT inputs are related to induction of learned avoidance behavior, whereas ST inputs are important for determining the direction to avoid. However, it has been reported that in *Helix*, orientation to learned attractive odors requires inputs from STs but not from ITs (Friedrich and Teyke, 1998). The findings for *Helix*, and those of the present study, suggest an interesting possibility that the contribution of ITs and STs to odor recognition is different for avoidance and attraction behavior, although there is no information on the function of each tentacle in learned attractive behavior in slugs. Recently, Ito *et al.* (1999) reported that electrical stimulation of the inferior and superior tentacle ganglion of *Limax* caused different responses in PC neurons, also suggesting that ITs and STs have different roles in olfactory processing in slugs.

Such a difference between the contribution of ITs and

STs to learned avoidance behavior raises the important and interesting question of how and where the inputs are integrated. It is well known that slugs avoid a conditioned odor and orient to the control side even if two different odor sources are presented on both sides (e.g. Sahley *et al*., 1981a, b, Sekiguchi *et al*., 1991). To complete this task, the slugs must pay attention to one of the two different odor gradients and use it for orientation. During this process, when the slug determines its behavior based on input from the IT system as shown here, the ST system — having an important role in orientation — would need to have information on the odors driving the IT system. Kimura *et al*. (1998b) have reported that afferent fibers from the IT tentacle ganglion innervate the terminal mass of the ipsilateral PC lobe where the axons from the ST tentacle ganglion also innervate. The PC lobe of the slug is considered to be involved in both olfactory recognition (Delaney *et al*., 1996, Kimura *et al*., 1998b, c) and learning (Kimura *et al*. 1998a). Thus, the PC lobe may also help to integrate the IT and ST inputs.

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