

Redundancy of olfactory sensory pathways for odor-aversion memory in the terrestrial slug *Limax valentianus*

Miki Yamagishi, Etsuro Ito and Ryota Matsuo*

Laboratory of Functional Biology, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Shido, Sanuki, Kagawa 769-2193, Japan

*Author for correspondence (e-mail: matsuo@kph.bunri-u.ac.jp)

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SUMMARY

Terrestrial slugs have the ability to learn and remember a food odor paired with an aversive stimulus. Olfaction in slugs involves the tips of two pairs of tentacles, the superior and the inferior tentacles. Sensory nerves in both pairs of the tentacles transmit olfactory information to the structure in the CNS, the procerebrum where learning and memory formation occur. We investigated the role of each pair of tentacles in odor-aversion learning, and examined the ability of slugs to recall memory after selective surgical amputation. Our results show that memory formation was not altered by the amputation of either one of the pairs before or after odor-aversion learning, while the odor sensibility of the slugs was maintained. These data suggest that either pair of tentacles is sufficient for the acquisition and retrieval of aversive olfactory memory.

Key words: *Limax*, odor-aversion learning, associative memory, superior tentacle, inferior tentacle.

INTRODUCTION

The terrestrial slug *Limax valentianus* has a highly developed olfactory system and is capable of associative odor-aversion learning (Gelperin, 1975; Sahley et al., 1981; Kasai et al., 2006). In this learning procedure, the slug is presented with the odor of a food such as carrot or cucumber juice whose taste has never been experienced by the slug. This odor serves as a conditioned stimulus (CS), and few slugs innately avoid this odor. However, once the odor is paired with an aversive stimulus such as the bitter taste of quinidine sulfate [the unconditioned stimulus (US)], the slug avoids the odor. The odor-aversion memory is established even by a single paired presentation of the CS and US (i.e. one-trial learning), and persists for at least two weeks (Nakaya et al., 2001; Matsuo et al., 2002).

Slugs perceive odor by two different pairs of tentacles: the longer ones called superior tentacles (STs) and the shorter ones called inferior tentacles (ITs; Fig. 1A). All four tentacles serve as olfactory organs, having olfactory epithelium on each of their tips. Several studies have reported differences in the roles of the two pairs. Using the terrestrial snail *Achatina fulica*, Chase and Croll (Chase and Croll, 1981) reported that STs are necessary for locomotion upwind toward a source of food odor, whereas ITs are necessary for trail following. Previously, we have demonstrated that the procerebrum (PC) is necessary for odor-aversion learning in *Limax* (Kasai et al., 2006). The PC receives olfactory sensory inputs from both STs and ITs (Chase, 1986; Gelperin et al., 1993; Chase and Tolloczko, 1993; Kimura et al., 1998). However, it is not known which sensory pathways, STs or ITs, are used during either the acquisition or the retrieval of olfactory aversive memory. It is possible that both sets are necessary for the acquisition and the retrieval of the memory, or it could be that a single pair is sufficient. A third possibility is that STs and ITs are involved differently; one used for acquisition and the other used for the retrieval. In the present study, we examined the roles of STs and ITs in the acquisition and the retrieval of odor-aversion memory by surgically amputating tentacle pairs.

Kimura et al. (Kimura et al., 1999) have previously investigated the effect of amputation of STs or ITs after olfactory conditioning on the retention and/or retrieval of olfactory aversive memory. They showed that IT amputation after conditioning degraded memory retrieval, whereas ST amputation had no such effect, suggesting that the olfactory inputs from the ITs were important for the retention and/or retrieval of aversive memory. However, there remain several points in their study to be evaluated again. First, they did not examine the effects of surgery on the ability of the slugs to sense the odor of the CS. If amputation resulted in an inability to sense the odor itself, then the conclusion that memory retrieval was altered would be unjustified. Second, there is a possibility that the acute tentacle amputation might result in abnormal behaviors, or in a reduction of mobility due to the physical stress. Third, they amputated tentacles only after conditioning, but not before conditioning. Therefore, we decided to re-examine the olfactory ability and the mobility of slugs following tentacle amputation. We also lengthened the recovery period following amputation, and as well performed tentacle amputation before conditioning.

We report here that either pair of tentacles (STs or ITs) is sufficient (and at least one pair is necessary) for the acquisition and retrieval of olfactory aversive memory. The functional redundancy of STs and ITs may confer a survival advantage to the slugs in its native environment.

MATERIALS AND METHODS

Animals

Terrestrial slugs *Limax valentianus* (Férussac 1822) (12–16 weeks post hatching) have been maintained in our laboratory at 19°C for at least nine generations as a closed colony. They have been fed only on a diet of humidified powder mixture consisting of 521 g rat chow (Oriental Yeast, Tokyo, Japan), 500 g wheat starch (Wako Pure Chemicals, Osaka, Japan) and 21 g vitamins (Oriental Yeast).

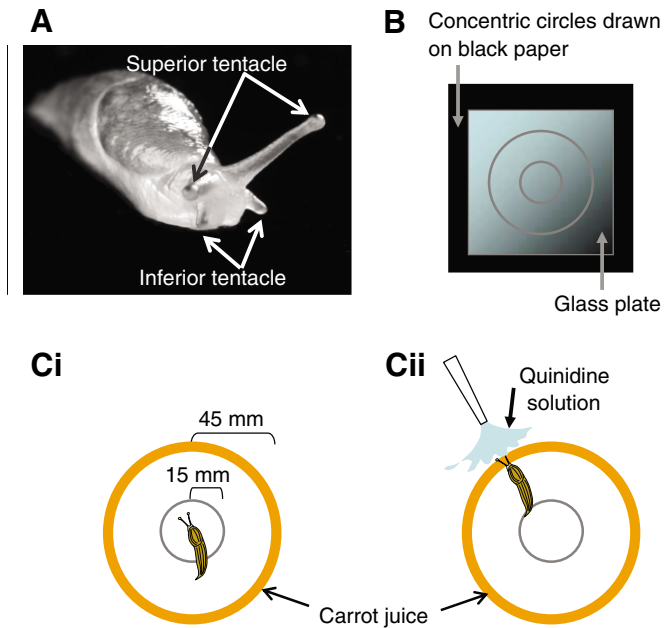


Fig. 1. Apparatus and procedure used in odor-aversion conditioning. (A) A view of a slug's head and tentacles. (B) The experimental apparatus. Concentric circles with radii of 45 mm and 15 mm were drawn on black paper, and a glass plate was superimposed on the paper. This was lit from above. (C) Procedure for odor-aversion conditioning. (i) Carrot juice (1 ml) was laid on the glass plate in the shape of the larger circle with a radius of 45 mm. A slug was placed on the center of the circle. (ii) In the paired conditioning, 1 ml of quinidine sulfate solution was applied to the mouth of the slug when it was just about to touch the carrot juice.

They have never eaten carrot or cucumber. All the behavioral experiments were performed in a room in which the air temperature and humidity were adjusted to 22°C and 60%, respectively.

Tentacle amputation

All the solutions injected into the slugs were sterilized by filtration through a syringe filter (Corning, New York, USA) immediately before injection. The slugs were anesthetized by an injection of approximately 250 μ l of ice-cold Mg^{2+} buffer (57.6 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} glucose, 2.36 mmol l^{-1} Hepes, 2.64 mmol l^{-1} Hepes-Na) into the body cavity. In the tentacle amputation, the middle parts of each pair of tentacles were cut with micro-scissors. For the slugs in the IT and ST+IT amputation groups, approximately 300 μ l of physiological buffer solution (70 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 4.7 mmol l^{-1} $MgCl_2$, 4.9 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} glucose, 2.36 mmol l^{-1} Hepes, 2.64 mmol l^{-1} Hepes-Na) was injected into the body cavity after the amputation to improve recovery from the surgery. In most cases, the wound healed spontaneously without any treatment. For the slugs in the control treatment group, only the physiological buffer solution was injected into the body cavity approximately 2 min after the injection of ice-cold Mg^{2+} buffer, without amputation of any tentacles. After surgery, each slug was transferred to a plastic container, and maintained individually with free access to the humidified powder mixture for 7 days until the next procedure, a conditioning or a memory retention test. The survival rate was monitored on the day after the surgery (Table 1).

Associative conditioning and memory retention test

The tentacle amputation was performed 7 days before or 1.5 h after the conditioning. We have previously performed behavioral

Table 1. The survival rate of the slugs monitored on the day after surgery

Amputated tentacle	Amputation	Saline injection	Survival rate (%)	Number of slugs
ST	Pre-conditioning	–	98.4	124
	Post-conditioning	–	87.5	80
IT	Pre-conditioning	–	100.0	68
	Post-conditioning	–	61.5	48
	Post-conditioning	+	100.0	70
ST + IT	Post-conditioning	+	98.6	72
Control	Pre-conditioning	+	100.0	62
	Post-conditioning	+	100.0	64

IT, inferior tentacle; ST, superior tentacle.

experiments with slugs using a shading box toward which the slugs are motivated to move (Matsuo et al., 2002; Kasai et al., 2006). We exploited their negative phototaxis in both the conditioning and the retention tests. The ST-amputated slugs, however, are deprived of their visual input because slugs have eyes on the tips of their STs (Fig. 1A), and thus this experimental system is not applicable. In the present study, we developed a new experimental system that does not use a shading box (Fig. 1B,C), to evaluate the roles of the STs and ITs in odor-aversion learning. Concentric circles with radii of 45 mm and 15 mm were drawn on black paper, and a glass plate was put on the paper. Carrot juice (1 ml) was put on the glass plate in the shape of a circle with a radius of 45 mm. The slug was then placed on the center of the circle. After the tip of the head crossed the first circle with the radius of 15 mm, the time count started. For the slugs in the paired conditioning group, 1 ml of saturated quinidine sulfate solution (Wako Pure Chemicals) dissolved in water was applied to the mouth of the slug when it was just about to touch the carrot juice. If the slug did not reach the carrot juice within 3 min after the tip of the head had passed the circle, the slug was not used for further experiments. After keeping the slug in contact with the quinidine solution for 90 s, the slug was submerged in water for 60 s. It was then returned to the plastic container, and was supplied with a humidified powder mixture of its usual food 1–2 h after the conditioning. The container was kept in an incubator at 19°C. The slugs in the unpaired conditioning group were treated in the same way except that only quinidine solution was applied 1 h after the presentation of the carrot juice odor. It has been demonstrated that a 1 h interval is sufficient to avoid forming any association of the two stimuli. The retention test was performed 24 h or 7 days after the conditioning, in the same apparatus that had been used in the conditioning. The slug was placed on the center of the circles. After the tip of the head crossed the circle with the radius of 15 mm, the time was counted until the slug touched the juice. If it touched the juice within 3 min after passing the 15 mm circle, it was judged to have lost its odor-aversion memory. If it showed hesitation (not touching the juice within 3 min), it was considered to have retained the memory. Avoidance (%) was defined as the relative number of slugs that did not reach the carrot juice within 3 min. One hour after the memory retention test with carrot juice, the mobility test was performed, in which the time was recorded to reach cucumber juice placed in the circle with a radius of 45 mm in the same way as in the retention test, to examine the odor specificity of the memory and to examine the mobility and the crawling speed of each slug. At least 1 h after the mobility test

with cucumber juice, the odor sensibility test (Kasai et al., 2006) was also carried out for the 21 slugs chosen at random from the post-conditioning IT amputation group (see below).

We carried out the conditioning and memory retention test for another group of slugs with IT amputation, using a shading box as described previously (Matsuo et al., 2002; Kasai et al., 2006). Briefly, the slug was conditioned on a glass plate shaded by a box, and the memory retention test was also performed in the same apparatus. In the memory retention test, 1 ml of carrot juice was put in the shape of a half circle with a radius of 90 mm, and the slug was placed just in front of the center of the circle. If it touched the juice within 3 min after passing the center toward the dark side, it was considered to have lost the odor-aversion memory. Otherwise, the slug was considered to retain the memory. The mobility test was also performed 1 h after the retention test by recording the time to reach the cucumber juice that had been laid in the shape of a half circle with a radius of 60 mm. All the retention tests were performed in a blinded manner; the experimenter was not told which slugs belonged to the paired or the unpaired conditioning group.

Odor sensibility test

The odor sensibility test was performed as described previously (Kasai et al., 2006) to assess the effects of the IT or ST+IT amputation on the ability of the slugs to sense the odor and to move normally. Briefly, a diluted humidified powder mixture of everyday food (for composition, see the section headed 'Animals' above) and a garlic homogenate were placed on a glass plate in the shape of half circles (5 cm radius) with each odor source on each half. The slug was gently placed on a glass plate in the center of the circle. The initial direction of the head was pointed toward the border of the two odor sources. Video movies made with a digital video camera recorder (Handycam, SONY, Tokyo, Japan) placed above (~70 cm) the plate provided the data for our analyses. The video recording continued until the slug reached either odor source. In the off-line analysis, the head position of the slug was plotted every 5 s, and the dots were connected by a line.

Section staining and image analysis

The tentacles of Pulmonata are known to have the ability to regenerate after amputation (Chase and Kamil, 1983). Histological analysis was performed as follows to determine whether the amputated tentacles of our subjects showed any sign of regeneration after the retention tests. One day following the completion of all the behavioral tests, a slug from the ST amputation group was chosen at random. An intact slug without surgery was also prepared. These slugs were deeply anesthetized by an injection of approximately 500 μ l of ice-cold Mg^{2+} buffer into the body cavity, and the STs were dissected out. They were frozen in Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan) with liquid nitrogen. Cryostat sections (10 μ m thick) were cut and mounted onto glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA, USA). The sections were dried for 30 min at room temperature, and then stained with 0.2% Toluidine Blue solution, and cover glasses were mounted with Eukitt (O. Kindler, Darmstadt, Germany). The images of the stained sections were obtained with a light microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (DP70, Olympus).

Statistical analysis

All data are expressed as mean \pm s.e.m. Differences between groups were examined for statistical significance using a χ^2 test and

Student's two-tailed *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

To investigate the roles of the STs and the ITs in odor-aversion learning in *Limax*, one or both (ST+IT) pairs of tentacles were amputated 7 days before or 1.5 h after the conditioning. We call the amputation before conditioning a 'pre-conditioning' amputation; and that after conditioning a 'post-conditioning' amputation. The survival rate of slugs 1 day after surgery is shown in Table 1. In the case of post-conditioning IT amputation, the survival rate was low without any treatment after surgery. The injection of a physiological buffer solution into the body cavity just after amputation increased the survival rate dramatically (Table 1). We therefore injected the physiological buffer solution into the slugs in the post-conditioning IT and ST+IT amputation groups, and also into the control groups.

Control experiments

Firstly, we investigated whether the new experimental system works. In the pre-conditioning control group (i.e. non-amputees), both the Mg^{2+} and physiological buffer solutions were injected without any surgical treatment. After 7 days, the slugs were conditioned, and the memory retention test was performed on the next day of the conditioning (Fig. 2Ai). In all memory retention tests described herein, the experimenters were blind with respect to the treatments being tested. In the paired conditioning group of slugs, 23 out of 31 slugs (74%) avoided the CS (carrot juice), whereas 12 out of 29 slugs (41.4%) in the unpaired conditioning group avoided the CS (Fig. 2Aii). This difference was statistically significant ($\chi^2=6.64$, $P<0.01$, d.f.=1 in all cases).

In the post-conditioning control group, the Mg^{2+} and physiological buffer solutions were injected 1.5 h after the conditioning, and the memory retention test was given 7 days later (Fig. 2Bi). In the paired conditioning group, 18 out of 32 slugs (56.3%) avoided the CS, whereas in the unpaired group, 9 out of 32 slugs (28.1%) avoided the CS (Fig. 2Bii). This difference also was significant ($\chi^2=5.19$, $P<0.05$).

These data show that odor-aversion memory is established in our new system, and that the injection of Mg^{2+} and physiological buffers does not affect the slugs' ability to learn and form memory. The pre-conditioning control group tended to show somewhat better memory retention than the post-conditioning control group (see Fig. 2Aii,Bii). This tendency is explained by the difference in the memory retention periods between the two groups (1 day *versus* 7 days) (Nakaya et al., 2001; Matsuo et al., 2002).

Amputation of both of the STs and ITs

We next determined whether the slugs could retain and retrieve odor-aversion memory in the absence of all four tentacles. All tentacles were amputated 1.5 h after the conditioning (Fig. 2Ci). After 7 days, the memory retention test was performed. In the paired conditioning group, 22 out of 35 slugs (62.9%) avoided the CS, whereas 15 out of 34 slugs (44.1%) in the unpaired group avoided the CS (Fig. 2Cii). There was no statistically significant difference in the avoidance rates ($\chi^2=2.44$, $P=0.119$) between the paired and the unpaired conditioning groups. These same slugs also showed high avoidance rates for cucumber juice, which was used as the control odor (Fig. 2Cii). We thus concluded that slugs that had all tentacles amputated after conditioning did not meet the necessary criteria to demonstrate memory retention of odor-aversion. We also tried amputating all tentacles before the conditioning. However, we could

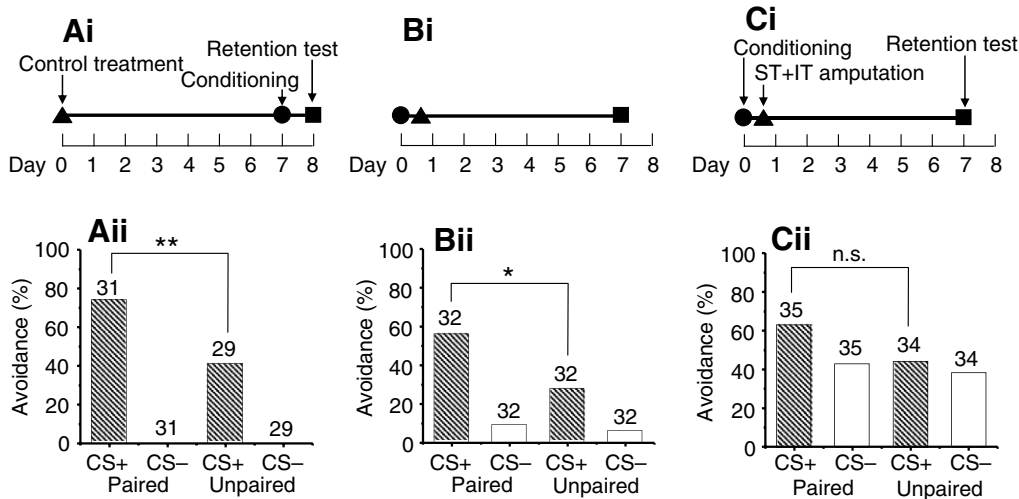


Fig. 2. Odor-aversion learning was not affected by the control treatment, but was completely abolished by amputation of both stets of tentacles, the STs and ITs. (Ai) Experimental schedule of the pre-conditioning control treatment. (Aii) The avoidance rate (%) of the slugs with the pre-conditioning control treatment. (Bi) Experimental schedule of the post-conditioning control treatment. (Bii) The avoidance rate (%) of the slugs with the post-conditioning control treatment. (Ci,Cii) Odor-aversion learning was affected by the amputation of both STs and ITs. (Ci) Experimental schedule of the post-conditioning ST+IT amputation. (Cii) The avoidance rate (%) of the slugs with the post-conditioning ST+IT amputation. The conditioning, the control treatment (or ST+IT amputation), and the retention test are indicated by ●, ▲ and ■, respectively. The numbers above the columns indicate the number of slugs used for the retention tests. * $P < 0.05$ and ** $P < 0.01$ by χ^2 test. n.s., not significantly different.

not successfully condition these slugs because a substantial number of the slugs (7 out of 14 slugs) did not reach the CS within 3 min during the conditioning.

Superior tentacle (ST) amputation

We next investigated the effects of amputation of a single pair of tentacles (either STs or ITs) on memory acquisition and memory retrieval. In the pre-conditioning ST amputation group, the conditioning was performed 7 days after surgery, and the retention test was given on the day following the conditioning (Fig. 3Ai). In the paired conditioning group, 59 out of 60 slugs (98.3%) avoided the CS, whereas 35 out of 59 slugs (59.3%) avoided the CS in the unpaired conditioning group (Fig. 3Aii). This difference was significant ($\chi^2 = 27.28$, $P < 0.01$). That is, the ST-amputated slugs demonstrated associative learning and memory.

In the post-conditioning ST amputation group, the surgery was performed 1.5 h after the conditioning, and the memory retention test was performed 7 days later (Fig. 3Bi). In the paired conditioning group, 30 out of 31 slugs (96.8%) avoided the CS, whereas in the unpaired conditioning group 16 out of 33 slugs (48.5%) avoided the CS (Fig. 3Bii). This difference also was significant ($\chi^2 = 18.45$, $P < 0.01$). We conclude that neither the acquisition nor the retrieval of odor-aversion memory was prevented by ST amputation. These results suggest that the ITs are sufficient to convey the necessary information for associative learning and the subsequent formation of memory to the CNS, as well as to convey odor information to the CNS necessary to retrieve the stored memory.

Inferior tentacle (IT) amputation

In the pre-conditioning IT amputation group, the slugs were conditioned 7 days after surgery and the memory retention test was performed on the following day (Fig. 4Ai). In the paired conditioning group, 14 out of 34 slugs (41.2%) avoided the CS, whereas in the unpaired conditioning group 3 out of 32 slugs (9.4%) avoided the CS (Fig. 4Aii). This difference was significant ($\chi^2 = 8.72$, $P < 0.01$).

In the post-conditioning IT amputation group, surgery was performed 1.5 h after the conditioning, and the memory retention test was performed 7 days later (Fig. 4Bi). In the paired conditioning group, 10 out of 38 slugs (26.3%) avoided the CS, whereas in the unpaired conditioning group 0 out of 30 slugs (0%) avoided the CS (Fig. 4Bii). This difference was significant ($P < 0.01$, by Fisher's exact probability test). We conclude that neither the acquisition nor the retrieval of odor-aversion memory was prevented by IT amputation.

However, whereas there was a statistical difference in the memory performance between the paired and unpaired groups, the

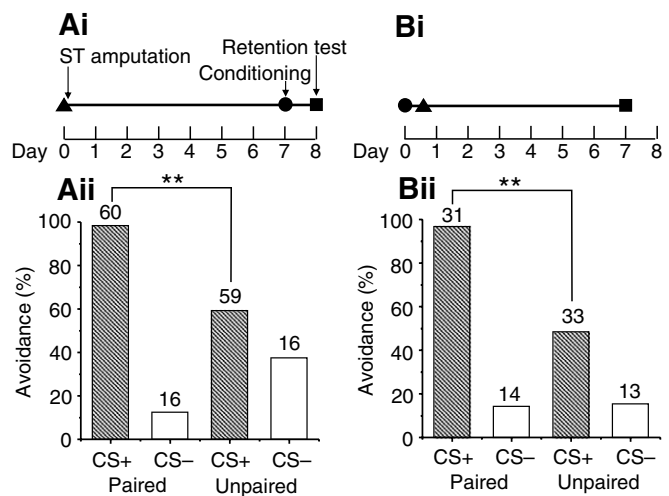


Fig. 3. Odor-aversion learning was not affected by ST amputation. (Ai) Experimental schedule of the pre-conditioning ST amputation. (Aii) The avoidance rate (%) of the slugs with the pre-conditioning ST amputation. (Bi) Experimental schedule of the post-conditioning ST amputation. (Bii) The avoidance rate (%) of the slugs with the post-conditioning ST amputation. The numbers above the columns indicate the number of slugs used for the retention tests. ** $P < 0.01$ by χ^2 test.

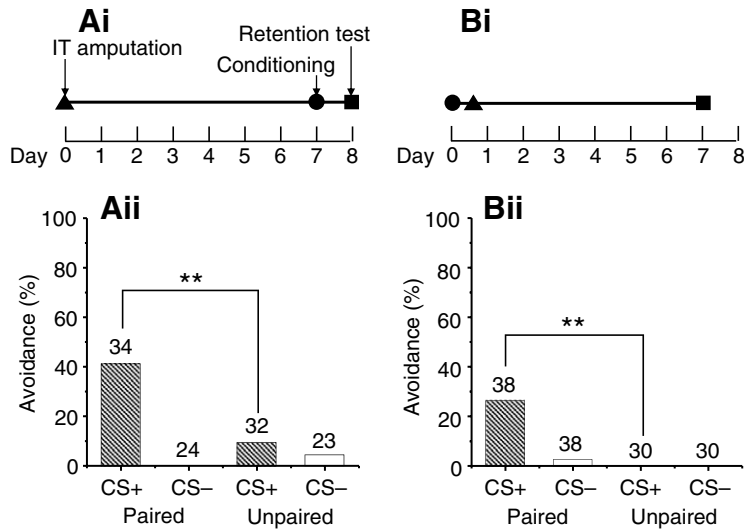


Fig. 4. Odor-aversion learning of the slugs with IT amputation. (Ai) Experimental schedule of the pre-conditioning IT amputation. (Aii) The avoidance rate (%) of the slugs with the pre-conditioning IT amputation. (Bi) Experimental schedule of the post-conditioning IT amputation. (Bii) The avoidance rate (%) of the slugs with the post-conditioning IT amputation. The numbers above the columns indicate the number of slugs used for the retention tests. ** $P < 0.01$ by χ^2 test (pre-conditioning amputation) or by Fisher's exact probability test (post-conditioning amputation).

avoidance rates were lower in both of the IT-amputated groups (pre-conditioning IT amputation group and post-conditioning IT amputation group) compared with the ST-amputated groups (Fig. 4Aii,Bii). This result might have been caused by some dysfunction in the odor sensing ability of the IT-amputated slugs. To test this possibility, we randomly chose 21 IT-amputated slugs (from the post-conditioning IT amputation group) immediately after the memory test (i.e. 7 days after the surgery), and monitored their behavior on a glass plate when they were surrounded by a circle

(5 cm radius) with a familiar odorant (everyday food) placed on one half, and an innately repellent odorant (garlic homogenate) on the other (Kasai et al., 2006). The 21 slugs consisted of 11 paired and 10 unpaired conditioned slugs. All the slugs (11 of 11 paired and 10 of 10 unpaired conditioned slugs) successfully reached their usual food (upper half) without touching the repellent odor source (Fig. 5). These data show that the ability to smell remained intact in the IT-amputated slugs and also their ability to regulate their crawling at the time of their memory retention test.

Although not shown here, in slugs that had both sets of tentacles amputated, 10 out of 21 slugs (47.6%) reached the garlic homogenate, indicating that amputation of all tentacles (STs+ITs) abolished their ability to sense and avoid an aversive odorant.

Conditioning and retention tests with a shading box

To confirm the capability of the IT-amputated slugs to learn and remember, we carried out a different type of conditioning and memory retention test using another group of slugs with the IT

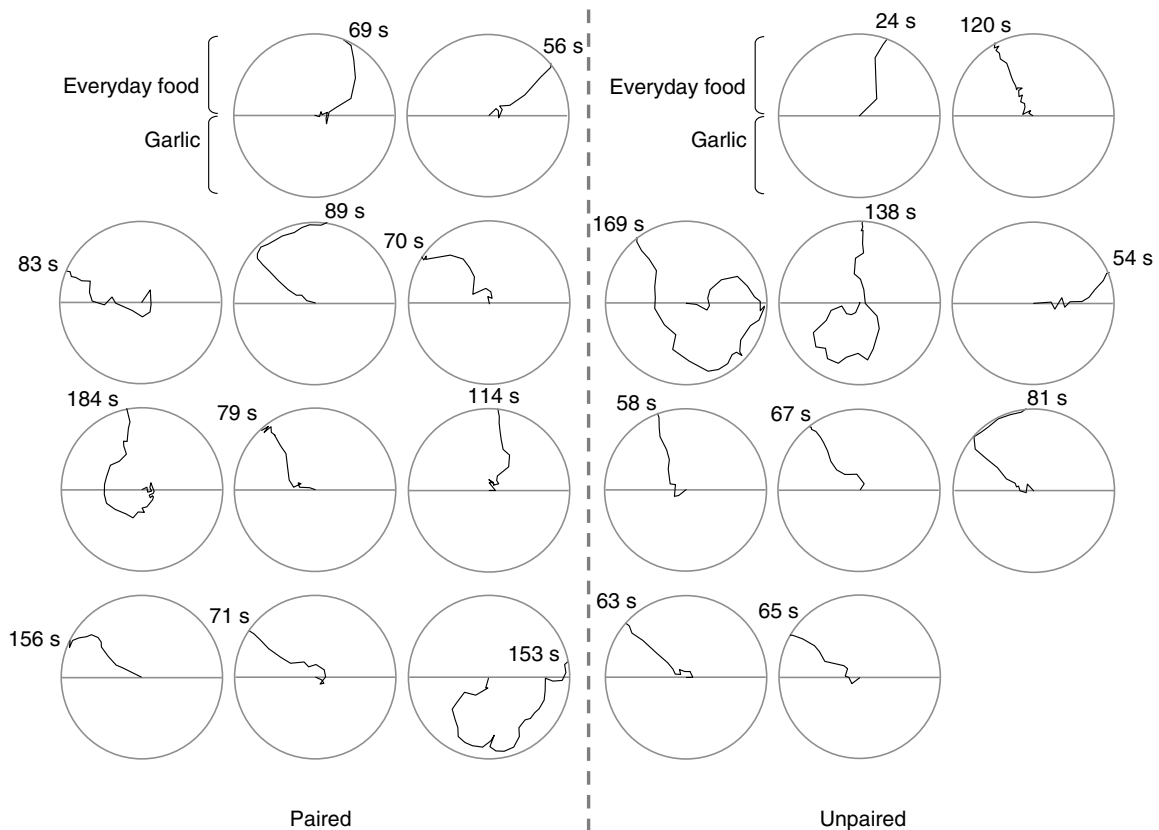


Fig. 5. Tracings of the paired and the unpaired conditioned slugs with IT amputation in the odor sensibility test. The humidified powder mixture of everyday food was placed on the upper half of each circle, and garlic homogenate was placed on the lower half. The time (in seconds) when the slugs reached either odor source is indicated. All slugs chose everyday food.

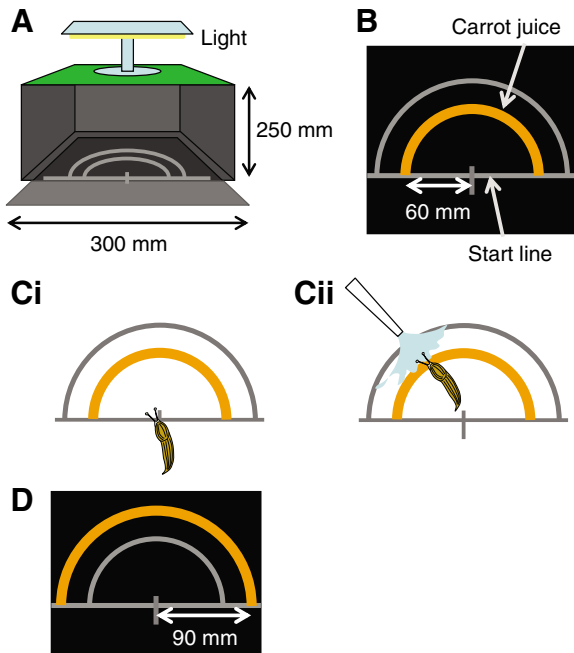


Fig. 6. Apparatus and procedure of odor-aversion conditioning with a shading box. (A) The experimental apparatus with a shading box. (B) During the conditioning, 1 ml of carrot juice was laid in the shape of a half circle with a radius of 60 mm. (C) The conditioning procedure. (i) The slugs were placed just behind the center of the start line. (ii) For the slugs in the paired conditioned group, 1 ml of saturated (approximately 1% w/v) quinidine sulfate solution was applied to the mouth of the slug when it was just about to touch the carrot juice. At the same time, we recorded the time it took to reach the carrot juice after the middle of the slug's body passed the start line. If the slug did not reach the carrot juice within 3 min, it was eliminated from the experiments. (D) In the retention test, the carrot juice was laid in the shape of a half circle with a radius of 90 mm, and we tested whether they avoided the carrot juice for at least 3 min.

amputation, using a shading box as described previously (Fig. 6A,B) (Matsuo et al., 2002; Kasai et al., 2006). This procedure exploits the slugs' negative phototaxis, and is applicable to IT amputation because the eyes remain intact. Halves of concentric circles with radii of 60 mm and 90 mm were drawn on black paper and a glass plate was placed on the paper, and this set was inserted into a shading box (Fig. 6A). During the conditioning, 1 ml of carrot juice (the CS) was placed on the glass plate in a circle with a radius of 60 mm (Fig. 6B,C). In the retention test, 1.5 ml of the CS was placed in a circle with a radius of 90 mm (Fig. 6D). A slug was judged to have lost its memory if it touched the carrot juice within 3 min.

The results of this memory test are shown in Fig. 7. In the pre-conditioning control treatment (Fig. 7Ai), 20 out of 24 slugs (83.3%) in the paired conditioning groups avoided the CS, whereas only 4 out of 24 slugs (16.7%) avoided the CS in the unpaired conditioning group (Fig. 7Aii). This difference was significant ($\chi^2=21.33$, $P<0.01$). These data confirmed that the previously used behavioral tests worked. However, the paired conditioned slugs in the pre-conditioning IT amputation group successfully avoided the CS [21 out of 24 slugs (87.5%)], whereas the unpaired conditioning slugs did not [9 out of 24 slugs (37.5%)] ($\chi^2=12.80$, $P<0.01$, Fig. 7Aiii).

In the post-conditioning control treatment group (Fig. 7Bi), 17 out of 24 slugs (70.8%) avoided the CS, whereas only 4 out of 24 slugs (16.7%) in the unpaired conditioning groups did (Fig. 7Bii).

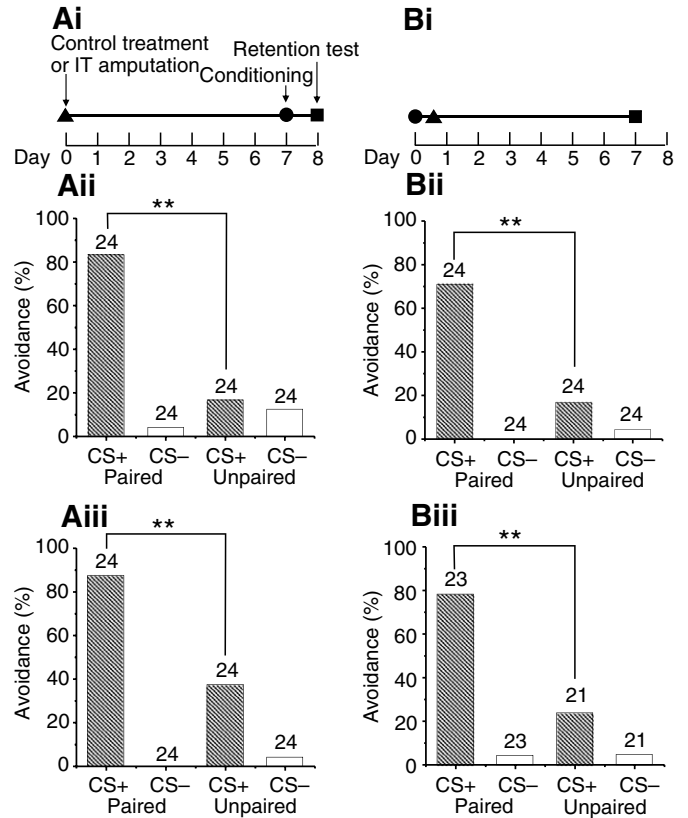


Fig. 7. Odor-aversion learning of the slugs with IT amputation, using a shading box. (Ai) Experimental schedule of the pre-conditioning control treatment or IT amputation. (Aii) The avoidance rate (%) of the slugs with the pre-conditioning control treatment. (Aiii) The avoidance rate (%) of the slugs with the pre-conditioning IT amputation. (Bi) Experimental schedule of the post-conditioning control treatment or IT amputation. (Bii) The avoidance rate (%) of the slugs with the post-conditioning control treatment. (Biii) The avoidance rate (%) of the slugs with the post-conditioning IT amputation. The numbers above the columns indicate the number of slugs used for the retention tests. ** $P<0.01$ by χ^2 test.

This difference was significant ($\chi^2=14.31$, $P<0.01$). In the post-conditioning IT amputation group, the paired-conditioned slugs also successfully avoided the CS (18 out of 23 slugs; 78.3%), whereas the unpaired conditioning slugs did not (5 out of 21 slugs; 23.8%; $\chi^2=13.05$, $P<0.01$; Fig. 7Biii). In this conditioning–retention test procedure, the avoidance rates of the paired groups were high (see Fig. 4Aii,Bii and Fig. 7Aiii,Biii), and the difference between the paired and the unpaired groups was more obvious than in the conditioning–retention test procedure in the lighter environment, confirming that the IT amputation did not affect the learning ability of the slugs.

Tentacle amputation did not affect the mobility of the slugs

To verify that tentacle amputation did not affect the mobility of the slugs, and that the memory is established only for the CS (carrot juice), we performed the mobility test using cucumber juice 1 h after the retention test for all or for randomly chosen slugs (Figs 2, 3, 4 and 7). The average time for the slugs to reach the cucumber juice is shown in Fig. 8A,B. Slugs that did not touch the cucumber juice within 3 min were excluded. We analyzed the difference between the paired and unpaired groups in each experiment. There

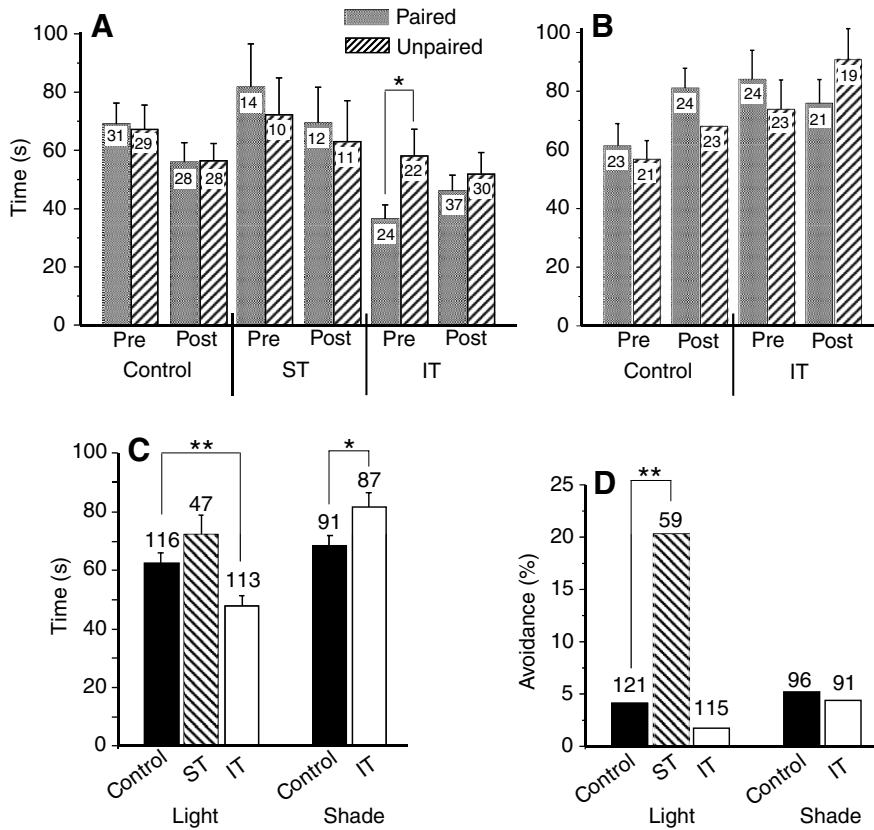


Fig. 8. The mobility test with cucumber juice. The average (\pm s.e.m.) time to reach the cucumber juice is shown as columns: ST, the superior tentacle-amputated group; IT, the inferior tentacle-amputated group. The numbers in or above the columns indicate the number of slugs used for that test. (A) Times taken in the lit environment (see Fig. 1). A significant difference was observed only in the pre-conditioning IT amputation group between the paired and unpaired-conditioned slugs. $*P < 0.05$ by Student's *t*-test. (B) Times taken under a shading box (Fig. 6). (C) Average time taken by slugs that underwent different surgical treatments to reach the cucumber juice in the lit or shaded environments. $**P < 0.01$, $*P < 0.05$ by Student's *t*-test. (D) The avoidance rates in the mobility tests with cucumber juice (> 3 min). $**P < 0.01$ by χ^2 test.

was no statistically significant difference except within the pre-conditioning IT amputation groups ($P < 0.05$, Student's *t*-test). The difference in the pre-conditioning IT amputation groups, however, does not alter the conclusion that the pre-conditioning IT amputated slugs could learn because the slugs in the unpaired group took longer than those in the paired group (Fig. 8A). This finding excludes the possibility that the higher rate of avoidance observed in the paired group (Fig. 4Aii) was caused by locomotory difficulties.

Next, we analyzed the difference in time to reach the cucumber juice between the groups that underwent different surgical treatments (Fig. 8C). In the experiments performed in the light environment, there was no difference ($P = 0.167$, Student's *t*-test) between the control and the ST-amputated groups, whereas the IT-amputated slugs moved faster toward the cucumber juice ($P < 0.01$, Student's *t*-test). By contrast, the IT-amputated slugs moved more slowly when tested in the experimental system in a shaded environment ($P < 0.05$, Student's *t*-test, Fig. 8C).

We also looked at the number of slugs that did not reach the cucumber juice within 3 min in the groups that underwent different surgical treatments (Fig. 8D). In the experiments performed in the light environment, there was a significant difference between the control and the ST-amputated groups ($\chi^2 = 12.18$, $P < 0.01$ by χ^2 test), whereas there was no difference between the control and the IT-amputated groups ($P = 0.447$ by Fisher's exact probability test). Similarly, there was no difference between the control and the IT-amputated groups in the experiments performed with a shading box ($P = 0.795$ by χ^2 -test, Fig. 8D).

Together these data suggest that the differences in the time to reach the CS between the paired and unpaired groups are not due to differences in mobility, but are the results of associative learning and memory formation.

Histological analysis

Histological analysis was performed to determine whether the amputated tentacles showed any signs of regeneration. One day after all the behavioral tests were completed (i.e. 8 days after surgery), two slugs were randomly chosen from the ST amputation group. Cryostat sections (10 μm thickness) were cut and stained with 0.2% Toluidine Blue solution. For comparison, stained sections taken from the intact slugs (Fig. 9Ai) were also examined. A tentacle nerve, a tentacle ganglion and an eye were discernible in the STs of the intact slugs (Fig. 9Aii, Aiii). By contrast, the tentacles of the ST-amputated slugs were still short (Fig. 9Bi), and the tips of the amputated STs were almost completely covered by a connective tissue-like structure without olfactory epithelium exposed to the air (Fig. 9Bii–v). It is known that tentacles of mollusks have a regenerative ability (Chase and Kamil, 1983) but as far as we could determine, no regeneration of tentacle ganglia or eyes had occurred.

DISCUSSION

We investigated the necessity for STs and the ITs in odor-aversion learning. In our odor-aversion learning paradigm, we measured the time during which the slug refrained from touching the carrot juice (Matsuo et al., 2002). We showed that neither amputation of the STs alone nor the ITs alone affects the learning ability of the slug, whether the amputation was performed before or after the conditioning. To achieve our aim, we introduced a novel experimental system for the behavioral assay. The results of the control groups clearly show that odor-aversion memory is established in our newly developed system, and that the injection of Mg^{2+} and physiological buffers, which served in the case of surgery as an anesthetic and a restorative, respectively, does not affect the slugs' ability to learn.

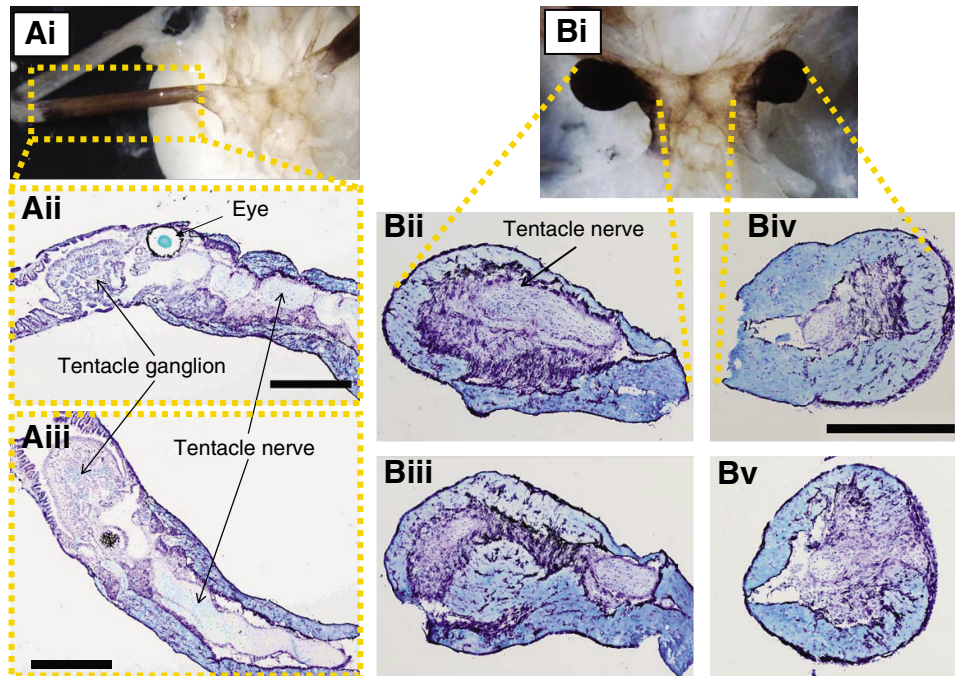


Fig. 9. Histological analysis of the tentacles of the slugs. Toluidine Blue-stained sections of the tentacles. (Ai) The brain and tentacles of an intact slug. (Aii,Aiii) Longitudinal sections of tentacles of intact slugs. (Bi) The brain and tentacles of a slug after an 8 day recovery period following ST amputation. (Bii–v) Longitudinal sections of the tentacles of slugs after an 8 day recovery period from ST amputation. Scale bars, 1 mm.

The pre-conditioning amputation experiments allow us to conclude that only a single pair of tentacles, either the STs or the ITs, is required for learning and memory formation. This means that a sufficient sensory neuronal signal about pairing of the CS-US can be conveyed to the CNS (i.e. the PC) with either set of tentacles, to bring about the necessary causal changes in neuronal activity that constitute learning and memory. The data obtained in post-conditioning amputation experiments allow us to further conclude that a single set of tentacles is sufficient to allow retrieval of a memory that was formed with both pairs of tentacles. It did not show any difference in the post-conditioning amputation experiments whether we amputated the STs or ITs. Thus, amputation of tentacles either before or after associative learning does not interrupt the formation of memory nor does it affect memory retrieval.

In the ST-amputated slugs, the aversion rates were notably higher than those of the controls, in both the paired and the unpaired groups (Fig. 3). Taking into account the fact that there was no difference in the time the ST-amputated and the control slugs took to reach cucumber juice during the mobility test (Fig. 8C), the high aversion rate in the ST amputation group does not seem to be caused by any difficulty in mobility. However, a higher rate of aversion to cucumber juice (>3 min) was observed in the ST amputation group (Fig. 8D). Although we do not have an explanation for these observations, it should be noted that ST amputation makes the slugs more cautious to approach any odor source, whatever the odorant is (Kimura, 2000). Because aversive behavior is selective for the conditioned odor in the intact slugs (Sahley et al., 1981; Nakaya et al., 2001), the tendency to avoid a general odor source would be unique to the ST-amputated slugs.

The results of the post-conditioning amputation experiments are intriguing because they show that the sensory representation created in the PC by either set of tentacles acting alone is sufficient to evoke the proper response (i.e. memory recall). Memory is context specific

(Haney and Lukowiak, 2001). That is, if the context is changed following learning and memory formation, the subject, when tested, responds as a naive subject, acting as though learning and memory formation had not occurred. One might have hypothesized that the sensory representation created in the PC during training with both sets of tentacles would be sufficiently different to the sensory representation conveyed by a single set of tentacles in the memory retention test session following amputation to cause the slug to act naively. Our data show that this was not the case: information conveyed by one set of tentacles was sufficient to trigger the memory formed with both sets.

It could also be argued that only one of the sets of tentacles carried the 'important' information necessary to create memory in PC neurons. Again, however, the data do not support this argument. Either set of tentacles was sufficient to encode learning and memory, as shown in the pre-conditioning amputation experiments. We therefore conclude that the sensory information supplied to the PC by either set of tentacles is of sufficient quality that it can elicit the proper memory retrieval.

The anatomy of both pairs of tentacles are consistent with our behavioral findings too. Both the tentacle nerves (in the STs) and the medial lip nerves (in the ITs) project to the PC, which is the locus where the neuronal changes underlying learning and the formation of odor-aversion memory occur (Kimura et al., 1998; Ermentrout et al., 2001; Kasai et al., 2006). Moreover, it has been demonstrated that the STs and the ITs have morphologically similar peripheral tentacle ganglia in the slug (Ito et al., 2000). Functional redundancy of two pairs of tentacles might be advantageous for the slugs, enabling them to survive injuries to some of their tentacles in the natural environment. Finally, since amputation of both pairs of tentacles abolishes learning, and even the ability to sense odor, it is clear that the tentacles play a sufficient and necessary role in odor-aversion conditioning.

Although our data demonstrated that the two pairs of tentacles are functionally redundant in respect to odor-aversion learning, does the slugs have two pairs only in preparation for injury? Can each pair substitute for the other's function whatever it is? It has been reported that each pair of tentacles serves a different function in some tasks other than odor-aversion learning: trail following is exclusively dependent upon the inferior tentacles, whereas orientation toward distant odor source depends on the superior tentacles (Chase and Croll, 1981). The dual tentacle pairs are a characteristic feature of the terrestrial Pulmonata, Stylommatophora (e.g. *Limax*), whereas the aquatic Pulmonata, Basommatophora (e.g. *Lymnaea*) has only a single pair of tentacles. From an evolutionary point of view, therefore, the origin of the dual tentacle pairs is not so ancient. Important functions such as the odor information transmission concerning aversive memory are shared by both pairs, whereas more diversified, terrestrial habitat-specific functions might be assigned to an either pair of tentacles.

Although we demonstrated the learning ability of the IT-amputated slugs, these data are not congruent with a previous report (Kimura et al., 1999) showing that post-conditioning amputation of IT abolished the ability to retain or retrieve odor-aversion memory. There are several possible explanations for this disagreement. First, they appeared to only perform the experiments in a light environment (as there was no mention of the use of a shading box). As we have seen here, IT-amputated slugs move faster for, as yet, unknown reasons (Fig. 8C), and the apparent avoidance rates then are reduced when tested in a light environment (Fig. 4Aii,Bii). These sorts of data might have misled Kimura et al. to conclude that the ITs were necessary for the retention/retrieval of odor-aversion memory. Second, the slugs were allowed to recover for 7 days after the surgery in our experiments, whereas they were only given a recovery period of several hours in the study of Kimura et al. A shorter recovery period, especially when IT amputation is concerned, almost certainly degrades performance. IT amputation is more traumatic on slugs than ST amputation (Table 1). Finally, we injected Mg^{2+} and physiological buffer solutions into the slugs in our amputation experiments. These solutions greatly reduced the traumatic side effects in our experiments.

Our data are also not consistent with another previous report. Friedrich and Teyke (Friedrich and Teyke, 1998) examined the roles of the inferior and the superior tentacles of the snail in food-attraction learning. They showed that the acquisition of olfactory memory requires sensory inputs conveyed by the ITs, whereas the recall of memory requires intact STs. These inconsistencies might be explained by differences in the learning paradigm used in the two studies. At its simplest, they used appetitive conditioning whereas we used aversive conditioning. Olfactory aversive and olfactory appetitive learning are thought to use different memory coding systems; the former requires serotonin for memory acquisition, whereas the latter does not (Teyke, 1996; Balaban et al., 1987; Shirahata et al., 2006). Thus, it would not be at all surprising that the obligatory sensory input pathways be different for aversive and appetitive learning.

Tentacle ganglia and tentacle nerves have an ability to regenerate after injury (Chase and Kamil, 1983). We included a 7-day post-surgery recovery period in our design so that the slugs could behave normally with respect to their diet and mobility. It is entirely possible that the amputated tentacles could have recovered their function during this period. Histological analysis, however, failed to detect any signs of regeneration of the tentacle ganglion or olfactory epithelium (Fig. 9). Moreover, olfaction-directed behavior was

completely disrupted by ST+IT amputation, indicating that the olfactory function of STs or ITs does not recover within 7 days. Finally, it has been reported that it took 10 weeks for the sensory function of the tentacles to return after the lesion in the terrestrial snail *Achatina fulica* (Chase and Kamil, 1983).

In the present study, the roles of each tentacle pair in the acquisition phase of memory could not be examined because the tentacles were removed irreversibly. In future studies, the temporary and reversible inactivation of tentacle function (e.g. use of lidocaine) will permit us to investigate the specific roles of each pair of tentacles during acquisition, maintenance or retrieval of odor-aversion memory (Friedrich and Teyke, 1998).

LIST OF SYMBOLS AND ABBREVIATIONS

CNS	central nervous system
CS	conditioned stimulus
IT	inferior tentacle
PC	procerebrum
ST	superior tentacle
US	unconditioned stimulus

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