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Olfactory Memory Storage and/or Retrieval Requires the Presence of the Exact Tentacle Used During Memory Acquisition in the Terrestrial Slug *Limax*

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Terrestrial pulmonates can form odor-aversion memories once a food odor is presented in combination with an aversive stimulus. Most of the olfactory information ascends via a tentacular ganglion located in the tip of the two pairs of tentacles, and is then transmitted to the higher olfactory center, the procerebrum. The procerebrum is the locus of memory storage and has been shown to be necessary for odor-aversion learning. However, it is unknown whether the procerebrum is the sole locus in which the memory engram resides. By exploiting the regenerative ability of tentacles, here we investigated whether tentacles function merely in transmitting olfactory information to the procerebrum, or constitute a part of the memory engram. We showed that after removal of the tentacles used during memory acquisition, slugs were unable to retrieve the memory, even if these tentacles were regenerated sufficiently to subserve memory function. Our results support the view that tentacles are more than conduits of odor information; they also participate in the formation of the memory engram.

Key words: tentacle, olfactory learning, regeneration, procerebrum, *Lehmannia*, Pulmonata

INTRODUCTION

Terrestrial slugs can form associative memories following simultaneous presentation of the odor of some food, such as vegetable juice, as a conditioned stimulus (CS), and an aversive stimulus, such as the bitterness of quinidine sulfate, as an unconditioned stimulus (US). Once such a memory is formed, slugs exhibit avoidance to the odor of vegetable juice, and the time to reach it is substantially increased. Because conditioning drives slugs avoid an odor that was not previously aversive, this type of memory is sometimes called odor-aversion memory. Odor-aversion memory can be established in a single conditioning session in slugs, and persists for several weeks (Sahley et al., 1981; Matsuo et al., 2002).

Olfactory information is detected by olfactory epithelia on the tips of the two pairs of tentacles, the superior tentacles (ST) and inferior tentacles (IT) (Fig. 1). Most olfactory information is thought to be processed in the primary olfactory center, called the tentacular ganglion (TG), which is located beneath the olfactory epithelia at the tip of each tentacle (Chase and Tolloczko, 1993). Information is then conveyed to the secondary olfactory center, the procerebrum (PC), located bilaterally at the side of the brain, although a minority of olfactory sensory neurons project directly to the PC (Chase and Tolloczko, 1993). Surgical destruction of the PC before conditioning abolishes the ability of the slug to acquire the odor-aversion memory without damaging odor detection, and post-conditioning destruction of the PC abolishes the retention and/or retrieval of the odor-aversion memory (Kasai et al., 2006), suggesting that the PC is required for this type of learning/memory.

Behavioral experiments exploiting the regenerative ability of the PC have further shown that the PC is involved not only in the acquisition/retrieval of the memory, but also in its storage (Matsuo et al., 2010a). This means that the memory engram resides in the neuronal network within the PC. However, it is unknown whether the PC is the sole locus of memory storage. It is possible that the memory engram also remains in other loci, such as the TG. If the memory is encoded in the neuronal networks encompassing the PC and the TG, it is expected that the same tentacles will be necessary for memory retrieval as were used during the memory formation.

Because the amputated tentacles are able to regenerate and regain their function following a recovery period (Chase and Kamil, 1983; Matsuo et al., 2010b; Matsuo and Ito, 2011), in the present study we sought to exploit this regenerative ability to examine whether or not the same tentacles must be used during the acquisition and retrieval phases in order for the memory to be retrievable.

MATERIALS AND METHODS

Animals

Terrestrial slugs *Limax valentianus* have been maintained in our laboratory at 19°C for at least 25 generations as a closed colony. They have been fed a continuous diet of humidified powder mixture (for its composition, see Fukunaga et al., 2006). All the slugs were used at the age of 3–3.5 months post hatching (adult, 0.2–0.8 g) at the start of the experiments.

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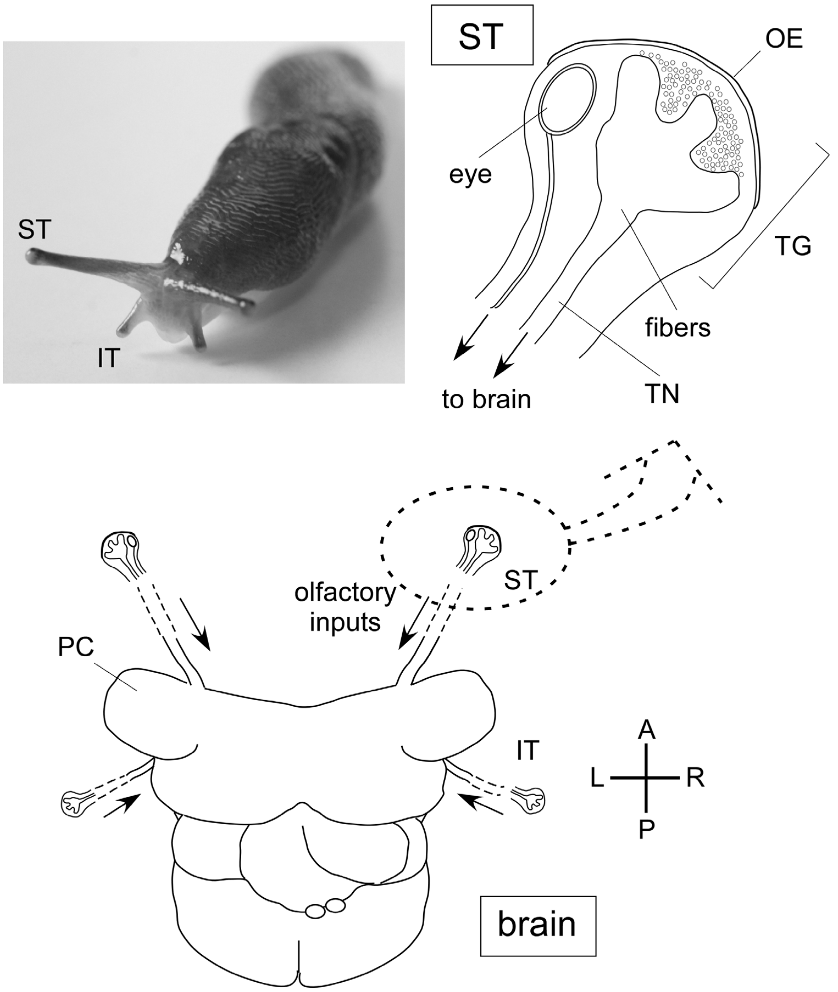

Fig. 1. Olfactory system of *Limax*. The locations of the ST and IT are shown in the photograph. Input pathways are depicted in the cartoon below, and anatomical structure of the ST is shown in the upper right. Both ST and IT are equipped with the TG, whereas only the ST possess an eye. Most of the olfactory information flows from the TG to the PC, which functions as a higher olfactory center. OE, olfactory epithelium; TN, tentacular nerve; A, anterior; P, posterior; R, right; L, left.

Surgical amputation of tentacles

Slugs (around 14 weeks post-hatching) were anesthetized by an injection of ice-cold Mg^{2+} buffer (57.6 mM $MgCl_2$, 5.0 mM glucose, 5.0 mM HEPES, pH 7.0) into the body cavity. To sustain the anesthesia, the slug was then laid on ice flakes for 1–2 minutes. Tentacle amputation was done under a stereo microscope. After amputation, approximately 500 μl of physiological saline (70.0 mM NaCl, 2.0 mM KCl, 4.7 mM $MgCl_2$, 4.9 mM $CaCl_2$, 5.0 mM glucose, 5.0 mM HEPES, pH 7.0) was injected into the body cavity to promote recovery from the anesthesia. Each slug was maintained individually in a plastic container, and food (humidified powder mixture) was supplied every 3–4 days.

Behavioral experiment

The slugs were conditioned and tested in an experimental setup that does not use a shading box, as described previously (Yamagishi et al., 2008) with slight modification. Briefly, the slugs were first divided into two separate groups; one is conditioned (conditioned group) and the other is not (control group). In the conditioning session,1 ml of carrot juice used as a CS was placed in the shape of a circle with a radius of 45 mm on a glass plate. A slug from the conditioned group was placed at the center of the circle. When the slug was about to reach the carrot juice, 1 ml of 1% (w/v) quinidine sulfate solution was applied as a US to the mouth of the slug, and the slug was kept in the mixture of the carrot juice and quinidine sulfate for 90 sec. (Slugs that did not reach the carrot juice within 3 min were not used for further experiments.) Each slug was then submerged in water and returned to its home plastic container after 60 s. In the memory retention test, 1 ml of carrot juice was again placed in the shape of a circle with a radius of 45 mm on the glass plate. The slug was placed at the center of the circle, and we recorded the time when the middle of the body crossed a circle with a radius of 15 mm. The slug was considered to retrieve the olfactory memory if it did not reach the carrot juice within 3 min, whereas it was considered to have lost the memory if it touched the juice within 3 min. All memory retention tests were done in a blinded manner, such that the experimenters did not know which slugs belonged to the conditioned group.

Toluidine-blue staining

Following the memory retention test (i.e. 35 days after amputation), the regenerated superior and inferior tentacles on the left side of the head were dissected out from the slug, which was deeply anesthetized with an injection of ice-cold Mg^{2+} buffer. The tentacles from age-matched slugs were also isolated as controls. Cryostat sections (14 μm-thick) were cut and mounted onto glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA, USA). Fixation and toluidine-blue staining were done as described previously (Matsuo et al., 2013). Images of the stained sections were obtained using a microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a CCD camera (DP-70, Olympus, Tokyo, Japan) and a ×10 (NA 0.30) objective lens.

Statistical analysis

Differences between the groups were examined for statistical significance using a χ^2 test or a Fisher's exact probability test; a significance level of $P < 0.05$ was considered significant.

RESULTS

Odor-aversion memory is long-lasting

In a previous study, we demonstrated that the amputated tentacles recover their normal olfactory functions within 34 days (Matsuo et al., 2010b). We thus tested whether the olfactory memory can be retained for a long enough time to allow the amputated tentacles to regenerate. Two groups of slugs were prepared: one was conditioned with carrot juice, and the other was not (the control group). The slugs were maintained individually for 36 days, and the retention of the memory was tested (Fig. 2A). Fourteen of 20 slugs avoided the carrot juice in the conditioned group, whereas only one of 20 slugs did in the control group. There was a statistically significant difference between the two groups (χ^2 = 18.03, P = 0.0001, Fig. 2B), which indicates that these slugs retain the odor-aversion memory for at least 36 days.

Regenerated tentacles function normally in olfactory learning

Previously, we have demonstrated that the slugs can acquire and retrieve olfactory memory using only regenerated superior tentacles (Matsuo et al., 2010b). We here explored whether the slugs can acquire and retrieve the memory using two pairs of regenerated tentacles that had been amputated 34 days prior to the conditioning. The memory retention was tested on the following day (Fig. 3A). Twenty-two of 25 slugs avoided the carrot juice in the conditioned group, whereas none of 24 slugs did in the control group ($\chi^2 = 38.33$, $P = 0.0001$, Fig. 3B). This result suggests that these slugs can accomplish olfactory memory tasks using only regenerated tentacles.

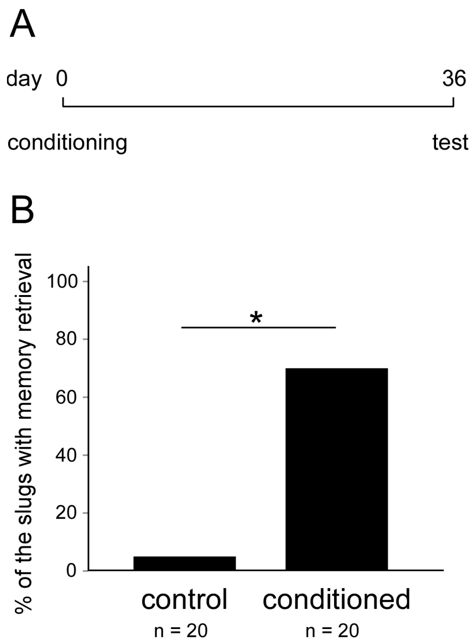

Fig. 2. Slugs can retain odor-aversion memory for 36 days. **(A)** Time schedule of the experiment. **(B)** The ratios (%) of the slugs that avoided the carrot juice were significantly different between the conditioned and control groups (*$P < 0.01$). The numerals below (*n*) indicate the number of animals used.

Olfactory memory cannot be retrieved with regenerated superior and inferior tentacles

We next investigated whether the odor-aversion memory acquired before the amputation of all the superior and inferior tentacles can be retrieved using the regenerated tentacles. Two days after the conditioning, both pairs of the superior and inferior tentacles were amputated, and the memory retention was tested after 34 days (Fig. 4A). None of the conditioned or control slugs avoided the carrot juice (0 of 17 and 0 of 11, respectively, $P = 1.0$ by Fisher's exact probability test, Fig. 4B), suggesting that the memory acquired before the tentacle amputation had been lost.

Structural recovery of amputated tentacles

Immediately after the memory retention test in the experiment described in Fig. 3 (i.e. 35 days following tentacle amputation), the structures of the regenerated superior and inferior tentacles were analyzed histologically. Both the control and regenerated superior tentacles were equipped with an eye, an optic nerve, a TG, and a tentacular nerve (Fig. 5A, B). Similarly, both of the control and regenerated inferior tentacles were equipped with a TG and a tentacular nerve (Fig. 5C, D).

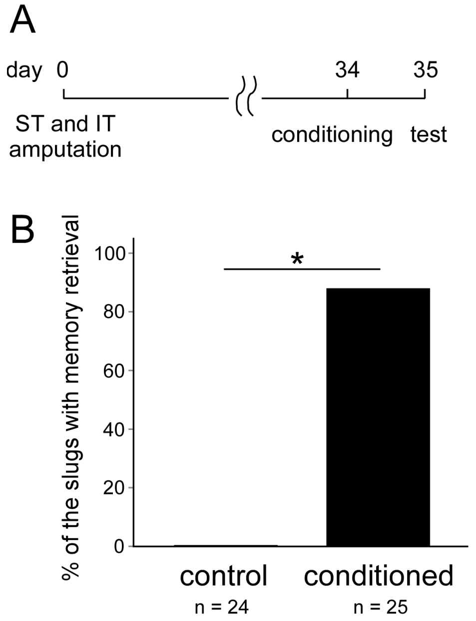

Fig. 3. Slugs can acquire and retrieve an odor-aversion memory using regenerated tentacles. **(A)** Time schedule of the experiment. **(B)** The ratios (%) of slugs that avoided the carrot juice were significantly different in the conditioned and control groups (*$P < 0.01$). The numerals below (*n*) indicate the number of animals used.

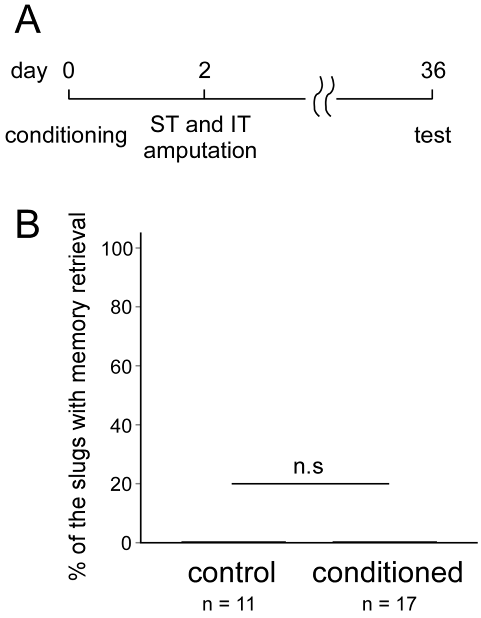

Fig. 4. Amputation of all the tentacles abolishes the memory even if they regenerate. **(A)** Time schedule of the experiment. **(B)** Neither the control or conditioned slugs avoided the carrot juice. The numerals below (*n*) indicate the number of animals used.

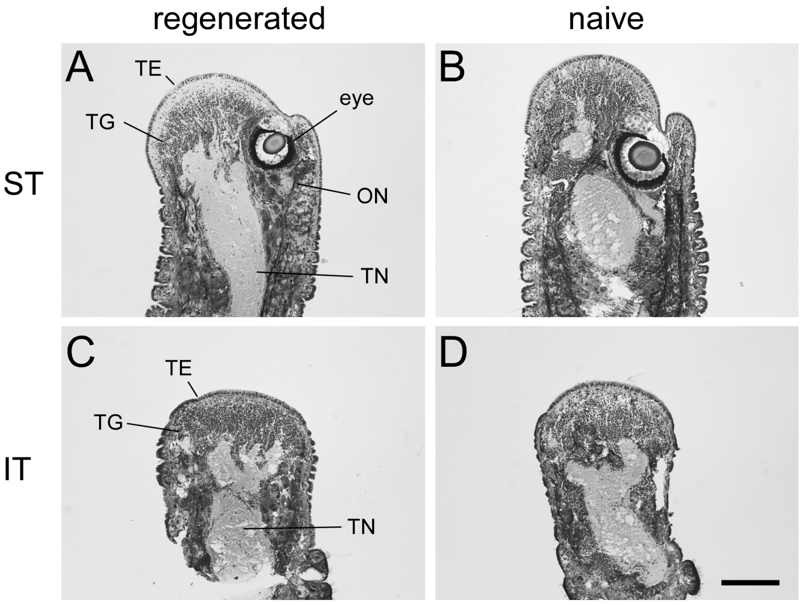

Fig. 5. Structures of the regenerated and naive tentacles. **(A)** A regenerated superior tentacle. **(B)** A naive superior tentacle. **(C)** A regenerated inferior tentacle. **(D)** A naive inferior tentacle. ST, superior tentacle; IT, inferior tentacle; TG, tentacular ganglion; OE, olfactory epithelium; ON, optic nerve; TN, tentacular nerve. Scale bar: 200 µm.

DISCUSSION

Stylommatophora pulmonates are equipped with two pairs of tentacles, STs and ITs. These serve as important sensory organs for multiple modalities, i.e. olfaction, vision, and somatosensation. Each tentacle also possesses a highly structured primary olfactory center called the TG at its tip, which contains numerous neurons with various morphological characteristics (Figs. 1, 5, Ito et al., 2000). In the TG, a field potential oscillation has been recorded in *Limax*, and its frequency is modulated by an exogenous application of several classical neurotransmitters (Ito et al., 2001; Inokuma et al., 2002; Ito et al., 2004). These characteristics are reminiscent of the PC, which is a well-known locus for the storage of olfactory memory (Gelperin, 1999; Watanabe et al., 2008; Matsuo et al., 2011).

In the present study, both superior and inferior tentacles recovered their original structures, including the eye, the optic nerve, olfactory epithelium, and the TG, 35days after the amputation (Fig. 5). Such morphological recovery seems to underlie the ability of slugs with only a pair of regenerated ST (Matsuo et al., 2010b) or pairs of regenerated ST and IT (Fig. 3), to accomplish an odor-aversion learning task.

In the present study, we demonstrated that the same tentacles as those used during the memory acquisition must be used during the memory retrieval for the memory to be faithfully retrieved. This suggests that the TG participate in encoding the representation of the olfactory information, and the memory engram is distributed within the neuronal networks encompassing the TG as well as the PC.

We previously showed that the memory acquired with two pairs of tentacles can even be retrieved using either one of the superior or inferior tentacles, and that the memory can be acquired and retrieved using either one of the superior or inferior tentacles (Yamagishi et al., 2008). These findings do not contradict our present results, because the same pairs of superior or inferior tentacles were functional both during the acquisition and retrieval of the memory in that study (Yamagishi et al., 2008). Friedrich and Teyke (1998), using an anesthetic agent, reported that the terrestrial snail *Helix* is unable to retrieve an appetitive olfactory memory if a different side (right or left) of the superior and inferior tentacles is allowed to function during memory acquisition and retrieval. This is also consistent with our present results, as their findings indicate that the same tentacles must be functional both during the acquisition and the retrieval of olfactory memory.

How does the TG participate in the memory engram? Inoue et al. (2004) reported intriguing data that may be informative here. They identified a neuronal pathway that evokes a mantle withdrawal response, one of the conditioned responses exhibited by a slug in the face of a learned odor. The mantle withdrawal is elicited through the serotonergic input to the TG from an identified serotonergic neuron, a posterior cerebral serotonergic cell (*p*-CSC) in the cerebral ganglion. In this case, retrieval of the memory (as evidenced by mantle withdrawal) inevitably requires the presence of the TG. However, it is unclear whether this pathway constitutes an odor-specific memory engram. It is possible that the neuronal pathway including the TG proposed by Inoue et al. (2004) may simply contribute to the expression of a general odor-induced aversive response.

To demonstrate the involvement of the TG in memory encoding more directly, it would be necessary to investigate physiological changes induced in the TG by memory formation. In fact, the PC exhibits a learned odor-specific change in the oscillatory frequency or amplitude of the spontaneous local field potential oscillation (Kimura et al., 1998a; Nikitin and Balaban, 2000; Inoue et al., 2006), and incorporation of Lucifer yellow dye into PC neurons specifically following odor-associative memory formation (Kimura et al., 1998b; Ermentrout et al., 2001; Sekiguchi et al., 2010). Future studies may reveal such discernible changes triggered in the TG by olfactory learning.

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