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Effects of tentacle amputation and regeneration on the morphology and activity of the olfactory center of the terrestrial slug *Limax valentianus*

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SUMMARY

The tentacles of pulmonates regenerate spontaneously following amputation. The regenerated tentacle is equipped with all the elements necessary for normal olfactory functioning, and the slugs can behave as well as they did before the tentacle amputation. However, it is not known what changes occur to the olfactory center procerebrum in the brain at the morphological and physiological levels. Here, we investigated the innervation of tentacular nerves into the procerebrum by examining the size of the terminal mass (input layer from tentacular nerves) of the procerebrum and also by staining afferent nerves immunohistochemically at 15, 58 and 75 days following unilateral amputation of the superior and inferior tentacles. The size of the terminal mass was significantly decreased, and the Phe-Met-Arg-Phe-NH_2ergic (FMRFamidergic) afferent nerves disappeared by 15 days following the tentacle amputation. However, the size of the terminal mass had recovered substantially by 58 days, as the tentacle regenerated. The FMRFamidergic innervation into the cerebral ganglion was also restored by this time. An extended recovery (75 days), however, did not result in any further increase in the size of the terminal mass. We also recorded the local field potential (LFP) oscillation in the procerebrum. We found that the oscillatory frequency of the LFP had decreased at 15 days following the tentacle amputation but had recovered at 58 and 75 days. These results suggest that the amputation and regrowth of the tentacle are accompanied by the respective degeneration and re-innervation of olfactory nerves, and these changes in the innervation status affect the basal state of LFP oscillation.

Key words: tentacle, degeneration, regeneration, *Limax*, procerebrum.

INTRODUCTION

The central and peripheral nervous systems of mollusks have a robust regenerative ability. The injured brain spontaneously regenerates its structure, and its function is restored without any exogenous treatment (Price, 1977; Moffett, 1995; Matsuo and Ito, 2010; Matsuo et al., 2010a). The most prominent example is the tentacle of terrestrial pulmonates (Stylommatophora). If the tentacle is amputated, a new one regrows spontaneously after a recovery period. The regenerated tentacle is equipped with a tentacle ganglion (Chase and Kamil, 1983), an eye (Eakin and Ferlatte, 1973; Flores et al., 1983) and has odor sensitivity comparable to that of an intact tentacle (Chase and Kamil, 1983). The serotonergic networks are also restored (Flores et al., 1992).

The tentacles send afferent inputs to the procerebrum (PC), the olfactory center involved in olfactory learning and odor discrimination (Kasai et al., 2006; Watanabe et al., 2008; Matsuo and Ito, 2010). The local field potential (LFP) recorded on the PC exhibits spontaneous oscillation (~0.7 Hz) induced by the synchronous activities of the inhibitory networks within the PC (Gelperin and Tank, 1990; Watanabe et al., 2008). It is thought that the oscillatory frequency of the LFP encodes the meaning/value of the odor that the tentacle detects (Kimura et al., 1998a; Chase, 2000; Inoue et al., 2006), and its change is thought to correspond to the decision-making that occurs just before the approach or avoidance of the odor sources (Samarova and Balaban, 2009). Both the superior and inferior tentacles innervate primarily to the terminal mass (TM) layer of the PC (Gelperin et al., 1993; Kawahara et al., 1997; Kimura et al., 1998b), where the olfactory information is transmitted to nonbursting neurons in the PC (Watanabe et al., 2008). It is not known, however, whether the tentacle amputation causes any morphological and/or electrophysiological changes in the PC.

In the present study, we investigated the effect of tentacle amputation and regeneration on the PC at the morphological level by examining the volume of the TM layer by means of NADPH-diaphorase staining at several time-points (15, 58 and 75 days) after unilateral tentacle amputation. We also examined the innervation of afferent inputs by immunohistochemical staining of the nerves containing Phe-Met-Arg-Phe-NH_2 (FMRFa) peptides that are thought to have projections from the tentacle (Suzuki et al., 1997; Kobayashi et al., 2010). Finally, we analyzed the LFP oscillation of the PC *in vitro* to assess the recovery of its electrophysiological activity.

MATERIALS AND METHODS

Animals

The terrestrial slugs *Limax valentianus* (Férussac 1822) were maintained in our laboratory at 19°C for 14–16 generations as a closed colony. They were fed on a diet of humidified powder mixture consisting of 521 g of rat chow (Oriental Yeast, Tokyo, Japan), 500 g of wheat starch (Wako, Osaka, Japan) and 21 g of vitamins (Oriental Yeast).

Surgery

The tentacle amputation of the slugs (12–13 weeks post hatching) was performed under anesthesia, as described previously (Yamagishi et al., 2008; Matsuo et al., 2010b). Both the superior and inferior tentacles were amputated unilaterally, and approximately 200 µl of physiological saline (70 mmol l^{-1} NaCl, 2.0 mmol l^{-1} KCl,

4.7 mmol l^{-1} $MgCl_2$, 4.9 mmol l^{-1} $CaCl_2$, 5.0 mmol l^{-1} glucose, 5.0 mmol l^{-1} Hepes, pH 7.0) was injected into the body cavity to improve the recovery from anesthetization. After surgery, two to five (usually three) slugs were transferred to a plastic container and maintained with free access to the humidified powder mixture until the next procedure – that is, *in vitro* LFP recording or histological analysis. To examine the volumes of the TM layer of the PC in naïve control animals, age-matched slugs were isolated in the same way and maintained with free access to the humidified powder mixture until sacrifice for anatomical examination.

Behavioral assay

Olfactory conditioning was performed as described previously (Matsuo et al., 2002). Memory retention was examined blindly with respect to whether the slugs had been conditioned or not one day earlier.

Electrophysiology

The slugs were deeply anesthetized by an injection of ice-cold Mg^{2+} buffer (57.6 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} glucose, 5.0 mmol l^{-1} HEPES, pH 7.0) into the body cavity, and their brains were dissected out in physiological saline that contained a high Mg^{2+} concentration (35.0 mmol l^{-1} NaCl, 2.0 mmol l^{-1} KCl, 28.0 mmol l^{-1} $MgCl_2$, 4.9 mmol l^{-1} $CaCl_2$, 5.0 mmol l^{-1} glucose, 5.0 mmol l^{-1} Hepes, pH 7.0). The LFP of the PCs was recorded in physiological saline for 1 min from the surface of the left and the right PCs simultaneously, using a glass suction electrode filled with physiological saline at room temperature. The LFP signal was differentially amplified and bandpass filtered at 0.1–100 Hz (MEG-2100, Nihon Koden, Tokyo, Japan). All recordings were made at room temperature (20–24°C).

Histological measurement of the terminal mass volume

The slug was deeply anesthetized by an injection of ice-cold Mg^{2+} buffer into the body cavity, and the brain was dissected out. The isolated brain was frozen in Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan) with liquid nitrogen. Serial cryostat sections (horizontal, 14 μm thick) were cut and mounted onto glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA, USA). NADPH-diaphorase staining was performed essentially as described previously (Matsuo and Ito, 2009), except that the concentrations of β-NADPH and nitroblue tetrazolium were both 0.1 mmol l^{-1} instead of 0.4 mmol l^{-1}. The images of the stained sections were obtained using a light microscope BX-51 with an attached cooled charge-coupled device (CCD) camera DP70 and an objective (×10, NA 0.40, Olympus, Tokyo, Japan). Because NADPH-diaphorase staining stains the internal mass (IM) layer of the PC most intensely (Gelperin et al., 2000; Matsuo and Ito, 2009), the border between the IM and the TM could be clearly defined. To calculate the volume of the TM, the area sandwiched between the cell mass (CM) layer and the IM layer was measured using the software CanvasX (Deneba, Victoria, BC, Canada) and was multiplied by 14 μm (thickness of the section), and then integrated throughout all the sections encompassing the terminal mass region (usually 20–30 sections).

Immunohistochemistry

The brain fixed for immunohistochemistry was prepared as described previously (Matsuo et al., 2009). Cryostat sections (coronal, 14 μm thick) were cut and mounted onto glass slides coated with Vectabond. After washing in diluted (by PBS to 5%) neutralized formaldehyde buffer (Nakarai-Tesque, Kyoto, Japan) for 5 min, the sections were immersed in PBS supplemented with 0.1% Triton X-100 (PBST). After blocking the sections using blocking buffer (PBST supplemented with 2.5% goat serum and 2.5% bovine serum albumin) at 4°C overnight, the sections were incubated in polyclonal anti-FMRFa antiserum (Neuromics, Northfield, MN, USA) diluted 1:3000 in blocking buffer for 1 h at room temperature. The sections were then washed in PBS, followed by incubation for 1 h with a secondary antibody against rabbit IgG labelled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), which was diluted 1:500 in blocking buffer at room temperature. After being washed in PBS, the sections were mounted in VectaShield with DAPI (Vector Laboratories). The fluorescence images were acquired with a fluorescence microscope (BX51; Olympus) equipped with a CCD camera (DP-70) and a ×10 (NA 0.40) objective lens. The specificity of the antiserum was confirmed by preabsorbing the antibody by an incubation of anti-FMRFa antiserum (diluted 1:3000 in blocking buffer) with 100 μmol l^{-1} FMRFa peptide.

Statistical analysis

All data are expressed as means ± s.e.m. For the analysis of the TM volumes, differences between groups were examined for statistical significance using a Student's two-tailed *t*-test or a Mann–Whitney non-parametric test. For behavioral studies, a χ^2-test was used. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The slugs were capable of learning five weeks after amputation of the superior tentacle

We first investigated whether the slugs were able to perform olfactory learning with their regenerated tentacles. The superior tentacles were amputated first, and the inferior tentacles were amputated four weeks later (Fig. 1A). In one group, the slugs were conditioned with carrot juice six days after amputation of the inferior tentacle. It has been demonstrated previously that the amputated tentacles do not regenerate in a recovery period as short as six days (Yamagishi et al., 2008). However, the superior tentacles, amputated 34 days before, showed regeneration to some extent at this time (R.M., unpublished observations). On the following day, their memory retention was tested [blinded with respect to the identities of the slugs (experimental or controls)]. As shown in Fig. 1B, all the slugs (10 of 10) successfully avoided the carrot juice, whereas a smaller portion of the other unconditioned slugs did (7 of 16). The difference was statistically significant (χ^2=8.60, P<0.005). This result indicates that the regenerated superior tentacles can subserve odor-aversion learning even after only a five-week recovery period, and functional connectivity exists between the tentacular ganglion and the PC at this time-point. Hereafter, we investigated the morphology and the activity of the PC during the course of tentacle regeneration.

The size of the TM layer changed following tentacle amputation

Both of the superior and inferior tentacles on one side were surgically amputated simultaneously. At 15 days after the amputation, the tentacles were still short. But, as reported previously (Chase and Kamil, 1983), the tentacles regenerated after a long recovery period [58 or 75 days after the amputation (Fig. 2, upper panels)], although the tentacles on the amputated side still had an undersized appearance. Surprisingly, the PC ipsilateral to the amputated tentacles was apparently smaller than the contralateral PC 15 days after amputation, whereas it was almost comparable after 58- and 75-day recovery periods (Fig. 2, lower panels).

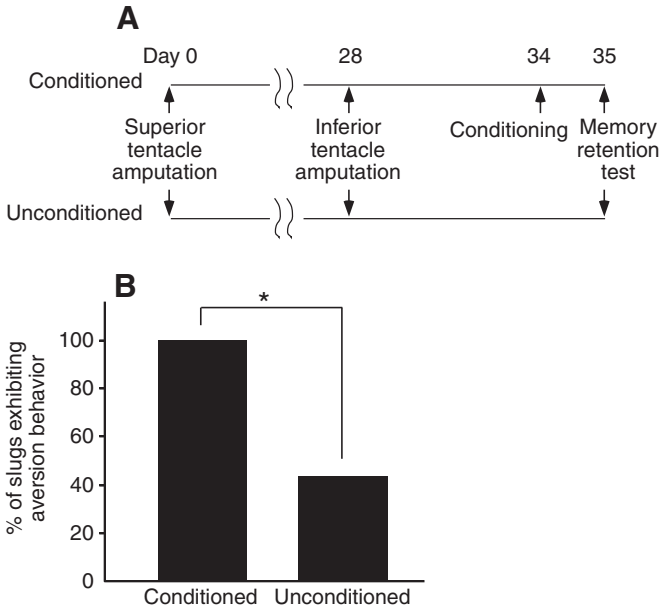

Fig. 1. The slugs could acquire and retrieve odor-aversion memory with regenerated superior tentacles. (A) Time schedule of surgeries and behavioral assay. (B) The percentage of the slugs that could avoid the conditioned odor. *P<0.005 by χ^2-test. N=10 for 'conditioned'; N=16 for 'unconditioned'.

Hereafter, we investigated quantitatively the morphology and the LFP oscillation of the PC as the tentacle regenerated.

To investigate the morphology of the PC in more detail, the brain was analyzed histochemically. We focused on the TM layer of the PC because the PC receives olfactory inputs from the tentacles mainly in this layer (Gelperin et al., 1993; Kawahara et al., 1997; Kimura et al., 1998b). To make visible the boundary between the TM layer and the IM layer, we exploited NADPH diaphorase staining, which is considered to reflect the enzymatic activity of nitric oxide synthase (Dawson et al., 1991). Because NADPH diaphorase staining stains the IM layer in the PC most intensely (Gelperin et al., 2000; Matsuo and Ito, 2009), we focused on the area sandwiched between the IM layer and the CM layer of the PC to define the TM layer. As shown in Fig. 3A, the TM layer was small on the side ipsilateral to the tentacle amputation 15 days after surgery. However, it was somewhat larger at 58 and 75 days after the surgery (Fig. 3A).

To analyze the sizes of the TM layers quantitatively, we measured their volumes. The areas of the TM were integrated throughout all the sections encompassing the whole TM layer to calculate the volume of the TM layer. In all the recovery groups, the volumes of the TM layer ipsilateral to the amputated tentacles were significantly smaller than those of the contralateral side [L *vs* R: L cut, P<0.005; R cut, P<0.001 in 15-, 58- and 75-day recovery groups (Fig. 3B; Tables 1 and 2)]. However, the relative volumes of the TM layer normalized to the contralateral side were significantly increased in the 58- and 75-day recovery groups (Fig. 3C). We also observed a tendency for the contralateral TM to become larger during the recovery period (Fig. 3B; Tables 1 and 2). This might be due to the growth of the slugs during the recovery period because we observed a similar tendency even in the naïve animals that had not undergone surgery [left TM of 15 days *vs* 75 days, P<0.05; right TM of 15 days *vs* 75 days, P<0.001 (Fig. 3D)].

Another interesting point is that the right TMs were smaller than the left TMs after recovery. The relative volumes of the TM ipsilateral to the amputated tentacles versus those contralateral (Fig. 3C) were substantially smaller in the right-tentacle-amputated slugs than in the left-tentacle-amputated slugs after recovery (58 days, P<0.001; 75 days, P<0.01). In fact, the actual volume of the TM ipsilateral to the amputation was larger in the left side than that in the right side at 58 days [$4.08(\pm0.25)\times10^7\,\mu m^3$ *vs* $3.39(\pm0.12)\times10^7\,\mu m^3$, P<0.05] although the difference was small at 75 days [$4.24(\pm0.20)\times10^7\,\mu m^3$ *vs* $3.96(\pm0.23)\times10^7\,\mu m^3$, P=0.39, Fig. 3B]. This does not seem to be due to the technical variability in surgical amputation of the tentacles because there was no difference in the actual sizes of the TMs at 15 days [P=0.094 (Fig. 3B)] and also because such an asymmetry is observed even in naïve animals (Fig. 3D).

The above results suggest that the tentacle amputation and regeneration accompany concomitant changes in the morphology of the PC, probably reflecting the afferent degeneration and re-innervation, respectively. To explore this possibility, the FMRFamidergic fibers were immunohistochemically stained in the

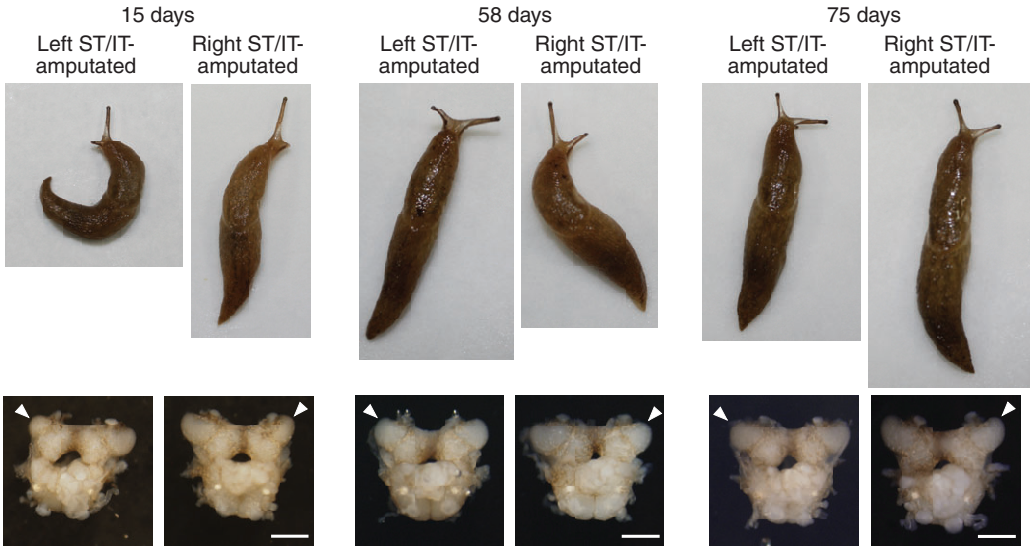

Fig. 2. Tentacle amputation resulted in apparent shrinkage of the ipsilateral PC. The size, however, was considerably restored after a long recovery period. Photographs were taken 15, 58 or 75 days following the tentacle amputation from different animals. Arrowheads indicate the PC ipsilateral to the amputated tentacles. Scale bars: 500 μm.

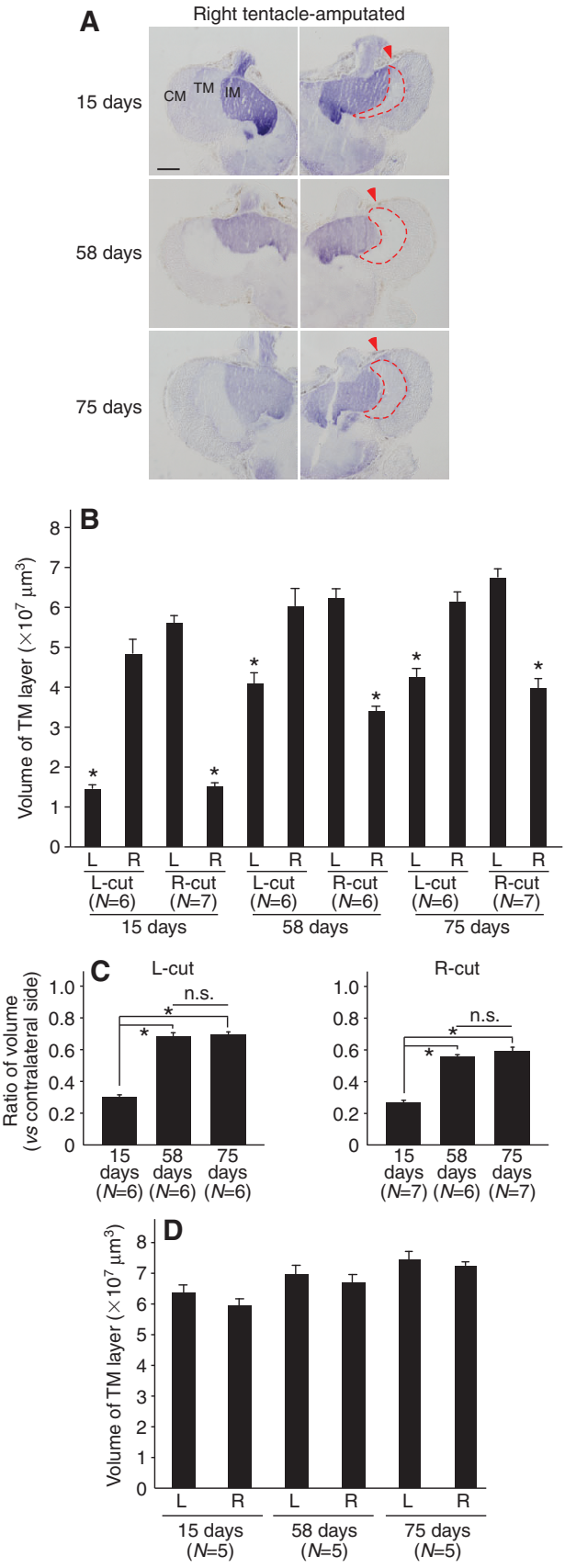

Fig. 3. The TM layer shrank after the tentacle amputation but regained its size substantially after a recovery period. (A) NADPH diaphorase staining of the PC 15, 58 and 75 days after amputation of the right tentacle. Red arrowheads indicate the TM layer ipsilateral to the tentacle amputation. The red dotted line delineates the TM. Scale bar: 200 μm. (B) The volumes of the TM layers. *P<0.005 *vs* contralateral side by Student's *t*-test or Mann–Whitney test. (C) The ratio of the TM volumes ipsilateral to the amputated tentacles relative to those contralateral to them. They were smaller in the right-tentacle-amputated slugs than in the left-tentacle-amputated slugs at 58 (P<0.001) and 75 (P<0.01) days. *P<0.01 by Student's *t*-test. (D) The volumes of the TM layers of naïve slugs maintained for 15, 58 or 75 days.

sections of the cerebral ganglion cut in the coronal direction (Fig. 4H). It has been demonstrated previously that FMRFamidergic primary sensory afferents ascend in the tentacular nerves to the cerebral ganglion, and these afferents disappear 14 days following amputation of the superior tentacle, possibly reflecting the nerve degeneration caused by tentacle amputation (Suzuki et al., 1997; Kobayashi et al., 2010). We could reproduce this result 15 days after the surgical amputation of the right superior and inferior tentacles (Fig. 4A,B). However, FMRFamide immunoreactivity was restored 58 and 75 days after the surgery (Fig. 4C–F). This result indicates that FMRFamidergic innervation regenerates during a 75-day recovery period. Preabsorption of primary antiserum by FMRFa peptide diminished the immunoreactivity (Fig. 4G).

Electrophysiological properties of the PC

To investigate the effect of the change in tentacular innervation on the activity of the olfactory center, we recorded the spontaneous LFP oscillation of the PC in the isolated brain. At 15 days following surgery, the oscillatory frequency was significantly reduced in the

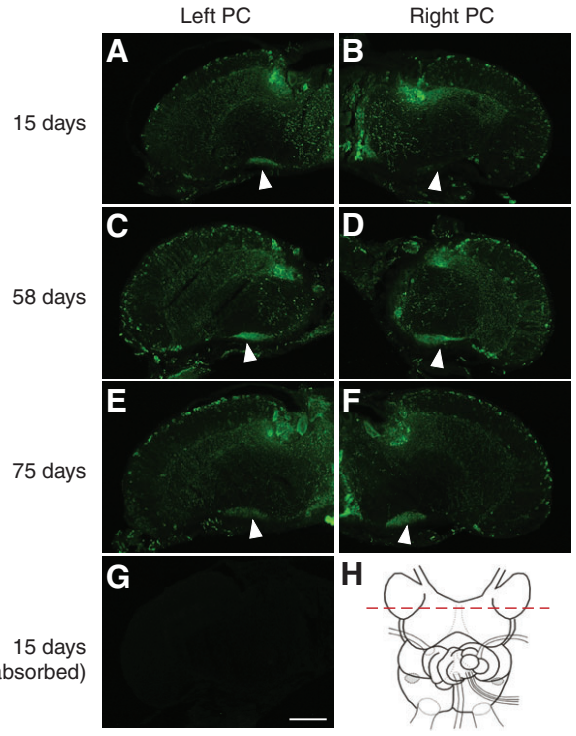

Fig. 4. FMRFa immunoreactivity was substantially reduced in the marginal area of the PC (arrowheads) ipsilateral to the amputated tentacles at 15 days after the surgery (A,B), whereas it reappeared at 58 and 75 days after the surgery (C–F). (G) Pre-absorption of the primary antiserum diminished the immune-positive signals. Scale bar: 200 μm. (H) A cartoon indicating the cutting plane (red dashed line) in the photographs of this figure.

Table 1. Pairwise comparison of the TM volume of left-tentacle-amputated slugs

	15 days		58 days		75 days	
	L	R	L	R	L	R
15 days L	–	<0.005	<0.001	–	<0.001	–
R		–	–	0.053 (n.s.)	–	<0.05
58 days L			–	<0.001	0.635 (n.s.)	–
R				–	–	0.840 (n.s.)
75 days L					–	<0.001
R						–

Numerals are *P*-values of Student's *t*-test or Mann–Whitney test. n.s.: not significant.

Table 2. Pairwise comparison of the TM volume of right-tentacle-amputated slugs

	15 days		58 days		75 days	
	L	R	L	R	L	R
15 days L	–	<0.001	<0.05	–	<0.001	–
R		–	–	<0.001	–	<0.001
58 days L			–	<0.001	0.097 (n.s.)	–
R				–	–	0.059 (n.s.)
75 days L					–	<0.001
R						–

Numerals are *P*-values of Student's *t*-test or Mann–Whitney test. n.s.: not significant.

PC ipsilateral to the amputated tentacles [left *vs* right PC of 15 days after L cut, $P<0.001$; left *vs* right PC of 15 days after R cut, $P<0.001$ (Fig. 5A,B)]. However, there was no difference in any of the other groups between the left and right PC [58 days after L cut, $P=0.26$; 58 days after R cut, $P=0.34$; 75 days after L cut, $P=0.56$; 75 days after R cut, $P=0.98$ (Fig. 5B)], suggesting that the spontaneous LFP oscillation was restored as the tentacle regenerated.

DISCUSSION

The nervous system of terrestrial mollusks exhibits a robust regenerative ability. One of the most prominent examples is the regeneration of tentacles by members of the Stylommatophora. Two pairs of tentacles function as multimodal sensory organs equipped with olfactory, mechanosensory and visual sensory organs and play important roles in food orientation behavior and trail following (Chase and Croll, 1981). Therefore, the damage or loss of either tentacle might threaten the survival of the animal, although the two pairs of tentacles are functionally redundant in odor-aversion learning (Yamagishi et al., 2008).

Chase and Kamil (Chase and Kamil, 1983) investigated the morphological aspects of tentacle regeneration focusing on the epithelial sensory pad of the tentacle ganglion in the superior tentacle of *Achatina fulica*. Consistent with their finding that odor sensitivity returns to normal levels by 10 weeks post surgery (Chase and Kamil, 1983), the LFP oscillation in the PC returned to the normal frequency by 58 and 75 days after the amputation (Fig. 5). This might reflect the restoration of a functional connection between the regenerated tentacular ganglion and the PC. In fact, the slugs could acquire and

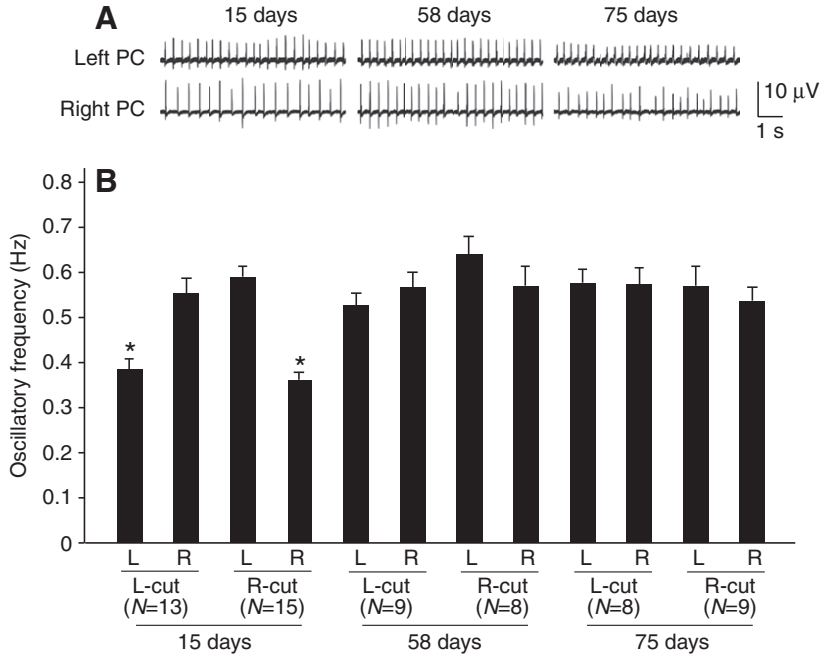

Fig. 5. The oscillatory frequency of the LFP was reduced in the PC ipsilateral to the tentacle amputation at 15 days after the surgery, whereas it recovered 58 and 75 days after the surgery. (A) Typical examples of LFP oscillation. (B) Quantitative data of oscillatory frequency (means ± s.e.m.). *$P<0.001$ by Student's *t*-test.

retrieve olfactory memory using their regenerated superior tentacles even five weeks post lesion (Fig. 1). In parallel, the volume of the TM layer, where the olfactory nerves from the tentacular ganglion project, was restored in parallel with the restoration of the LFP oscillation in the PC. The degeneration/regeneration of afferent projection to the cerebral ganglion was further supported by immunohistochemical analysis of FMRFa (Fig. 4), although FMRFamidergic afferents do not directly enter into the TM layer of the PC (Kobayashi et al., 2010). Unfortunately, the identity of the neurotransmitter that is released from the projection nerves in the TM layer of the PC has not been determined. Altogether, our present results demonstrate that the functional regeneration of tentacles accompanies concomitant morphological and electrophysiological changes in the PC.

The sensory pad of the tentacular ganglion shows regeneration very slowly at the morphological level. It continues to reconstruct itself up to 20 weeks post amputation (Chase and Kamil, 1983). By contrast, we did not observe further enlargement of the TM layer beyond 58 days post surgery (Fig. 3C). Moreover, the regenerated tentacle had an undersized appearance even at 75 days post surgery (Fig. 2), consistent with a previous report (Chase and Kamil, 1983). These facts suggest that the slugs/snails do not restore their organs exactly to the original status, but rather they can manage to perform olfaction-based tasks using their incompletely regenerated olfactory organs.

Another interesting observation made in the present study is that the final size of the TM layer was smaller on the right side than on the left side (Fig. 3C). Even in the naïve animals, there was a tendency for the right TM layer to be smaller than the left TM layer, although there were no statistically significant differences (Fig. 3D). As far as we know, such morphological asymmetry has not been reported in bilateral PCs. This characteristic might be due to asymmetry of the mesocerebrum located near the PC in the cerebral ganglion (Chase, 2000).

Currently, we cannot explain why the frequency of the LFP oscillation was downregulated by deafferentation. In our experimental system, there was no physiological sensory input from the tentacles because the brain was isolated from the remaining parts of the body during recording. Therefore, the downregulation of the frequency does not seem to be caused by the absence of sensory inputs. Continuous spontaneous inputs from the terminals of tentacular nerves might normally raise the basal activity of the neural networks within the PC, and the loss of this input results in a reduction of the oscillatory frequency. As it is not known what kind of neurotransmitters mediate the tentacular input to the PC, biochemical investigation is awaited to elucidate the mechanism of the downregulation of oscillation by deafferentation.

LIST OF SYMBOLS AND ABBREVIATIONS

CCD	cooled charge-coupled device
CM	cell mass
DAPI	4′,6-diamino-2-phenylindole
FMRFa	Phe-Met-Arg-Phe-NH_2
IM	internal mass
LFP	local field potential
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate-buffered saline
PC	procerebrum
TM	terminal mass

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