Analysis of spontaneous regeneration of olfactory structures with emphasis on myelination and re-innervation of cortical areas

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ABSTRACT

Regeneration of the lateral olfactory tract (LOT) occurs spontaneously after transection in developing rats. In neonatally LOT-transected rats, we observed a newly-formed myelinated tract near the rhinal sulcus. The aim of this study was to analyze the precise re-innervated cortical areas and to demonstrate ectopic LOT myelination in neonatally LOT-transected rats. Neonatal rats were subjected to unilateral LOT transection and simultaneous injection of a retrograde fluorescent tracer into the posterior olfactory cortex to evaluate the degree of transection. After 8 weeks, bilateral olfactory bulbs of the rats were subjected to multiple injections of an anterograde neuronal tracer to determine the extent of the regenerated fibers. In the completely LOT-transected rats, the regenerated fibers were distributed in the anterior olfactory cortices; the anterior olfactory nucleus, the olfactory tubercle, and the rostral part of the piriform cortex. Ectopic myelination of LOT was evident immediately below the rhinal sulcus in the completely and incompletely LOT-transected rats. We concluded that the regenerated bulbar fibers were confined to the regions of the anterior olfactory cortices and that ectopic myelination of the regenerated LOT occurred only at a specific site near the rhinal sulcus.

Keywords: Central olfactory tract; Mitral cells; Neuronal tracing; Regenerated areas; Ectopic myelination; Neonatal rats

1. Introduction

The lateral olfactory tract (LOT) is the main fiber tract of the central olfactory system and connects the olfactory bulb to multiple ventrobasal cortical areas, which are collectively known as the olfactory cortices [1,7,13,14,17,18]. Generally, LOT is macroscopically observed as a white myelinated band on the basal surface of the brain in adult rats. Recently, we reported spontaneous LOT regeneration and functional recovery in developing rats [15]. The most striking feature of the regenerated olfactory structures was an almost total lack of myelination in the regenerated LOT, which we initially misinterpreted as absence of LOT regeneration. To demonstrate the unequivocal LOT regeneration, we analyzed completely LOT-transected rats using a retrograde fluorescent tracer that was simultaneously injected at the LOT transection to exclude the incompletely LOT-transected rats. However, we observed that the newly-formed myelinated tract was located outside the original LOT in the LOT-transected rats. Therefore, the relation between the extent of LOT injury and myelination in the original and ectopically regenerated LOTs was the focus of the current study.

Previously, we provided a brief description of the extent of the original distribution of regenerated bulbar projections in the olfactory cortices [15]. However, the precise extent of re-innervated olfactory cortices that receive afferent fibers from the olfactory bulb still requires clarification. Therefore, the present study analyzed the spontaneously regenerated olfactory structures in greater detail with an emphasis on myelination and the extent of re-innervated cortical areas, demonstrating that neonatally LOT-transected rats exhibited varying degrees of LOT damage at the adult stage.

2. Materials and Methods

2.1. Animals

This study used 15 newborn Wistar rat pups of both sexes (Japan SLC Inc., Hamamatsu, Japan). Postnatal day (P) 0 refers to the first 24h after birth. All procedures were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by our Institutional Animal Care and Use Committee. All efforts were made to minimize animal numbers and their suffering. Surgical procedures were performed under general anesthesia with hypothermia (−20°C, 15 min) at the neonatal stage. At the adult stage, rats were anesthetized by an intraperitoneal injection of a mixture of pentobarbital (50 mg/kg) and medetomidine (10 mg/kg). Antipamezole hydrochloride (2 mg/kg, intraperitoneally) was injected to reverse the anesthesia.

2.2. LOT transection and injection of retrograde fluorescent tracer at the neonatal stage

P2 pups were subjected to left unilateral LOT transection by inserting the tip of a knife (Ophthalmic Scleral MVR Knife, 25 gauge; Alcon, Tokyo, Japan) through an approach path that was created from the ventrolateral aspect of the head between the eye and the ear. Immediately after LOT transection, 0.1 μl of 1% Fast Blue (FB) (Polysciences Inc., Warrington, PA, USA), a retrograde fluorescent tracer, was injected into the posterior olfactory cortex (the olfactory tubercle and the piriform cortex), which was situated far caudal to the site of the LOT transection. After surgery, the pups were housed with their dam in a single cage (26 × 42 × 18 cm) under standard laboratory conditions with a 12-h light/dark cycle and a room temperature maintained at 22 ± 1 °C. The pups could move freely within their cages, and food and water were supplied *ad libitum*.

2.3. Anterograde neuronal tracing at the adult stage and tissue preparation

LOT-transected and FB-injected neonatal rats survived until 8 weeks. The olfactory bulbs on both sides were subjected to 5 injections of 0.2 μl of an anterograde neuronal tracer, biotinylated dextran amine (BDA) (BDA-10000; Molecular Probes, Eugene, OR, USA). After 3 days, the rats were deeply anesthetized with sodium pentobarbital (80–100 mg/kg, intraperitoneally) and perfused through the heart with 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The rat brains were removed, post-fixed overnight in the same fixative, soaked in 30% phosphate-buffered sucrose for 2 days, and divided into 2 regions (olfactory bulbs and other brain regions). Using a freezing microtome, the olfactory bulbs were cut into a series of 50-μm slices in the sagittal plane at 300-μm intervals and the other brains were cut into a series of 50-μm slices in the coronal plane at 300-μm intervals. The first set of sections were mounted as a series on coated slides, cover-slipped with glycerol, and observed under a fluorescence microscope.

2.4. BDA detection and immunohistochemistry

The second set of sagittal bulbar and coronal brain sections were processed for BDA detection. After immersion in 0.3% H_2O_2 for 30 min to suppress endogenous peroxidase activity, the sections were incubated for 2 h in 0.1 M phosphate-buffered saline containing an avidin-biotin-peroxidase complex (1.5%) and Triton X-100 (0.3%). The samples were visualized with a Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific Inc., Pierce Biotechnology, Rockford, IL, USA). The sections were rinsed, mounted on coated slides, air-dried, dehydrated, and cover-slipped with Entellan New

(Merck, Darmstadt, Germany).

The third set of coronal sections were immunostained using an anti-myelin basic protein (MBP) antibody to detect the myelin sheaths. The sections were incubated overnight with mouse anti-MBP antibody (1:10000; Protos Biotech Corporation, New York, NY, USA). After washing, they were incubated with Alexa 488-conjugated donkey anti-mouse secondary antibody (1:1000; Molecular Probes) for 2 h. The sections were rinsed, mounted on coated slides, cover-slipped with glycerol, and observed under a fluorescence microscope.

3. Results

In the completely LOT-transected cases (n = 5) where no FB-positive mitral cells were found in the olfactory bulb on the left transected side, macroscopic examination revealed no white myelinated bands at the original LOT position (Fig. 1A–C). However, microscopic examination revealed the presence of thin MBP-positive myelinated components in the outer superficial layers of the olfactory tubercle and the rostral part of the piriform cortex. Of particular interest was the constant occurrence of the newly-formed myelinated fiber bundles immediately below the rhinal sulcus. These ectopic LOTs were confirmed by macroscopic examination and MBP immunohistochemistry (Fig. 1A, D, and E). Fig. 1H–J show the bilateral BDA-positive bulbar terminal regions situated ventrolaterally in a rostrocaudal sequence. On the right uninjured side, the BDA-positive bulbar fibers and terminals were visible at the most caudal end of the posterior olfactory cortex. However, the BDA-positive bulbar fibers and terminals on the left transected side were distributed in approximately anterior two-thirds of the olfactory cortex including the anterior olfactory nucleus, the olfactory

tubercle, and the rostral part of the piriform cortex; they did not extend caudally to the posterior olfactory cortex. MBP-positive myelinated components constituted the thin outer layer of the BDA-positive olfactory cortex.

In the incompletely LOT-transected cases (n = 10), macroscopic examination revealed white narrow myelinated bands of various sizes at the original LOT position on the left transected side and microscopic examination revealed a substantial number of FB-positive mitral cells in the left olfactory bulb (Fig. 2A–C). FB-positive mitral cells with wider residual LOTs were more abundant than those with narrower residual LOTs. In the incompletely LOT-transected cases, the newly-formed myelinated fiber bundles (ectopic LOTs) were visible immediately below the rhinal sulcus in addition to the original LOTs (Fig. 2A, D, and E). BDA-positive bulbar fibers and terminals on the left transected side were observed throughout all parts of the olfactory cortex, which was remarkably similar to the distribution observed on the right uninjured side (Fig. 2H–J). Fig. 3 shows the distributions of the BDA-positive bulbar fibers and terminals of the completely and incompletely LOT-transected cases.

4. Discussion

LOT injuries were classified as complete or incomplete transection based on neuronal labeling of the bulbar projection neurons (mitral cells) with a retrograde fluorescent tracer (FB) that was injected immediately after LOT transection. In the completely LOT-transected rats, BDA-positive bulbar fibers and terminals were observed in approximately anterior two-thirds of the olfactory cortex and they contained truly regenerated axonal elements; FB-positive mitral cells were absent in the olfactory bulb. In the incompletely LOT-transected rats, a variable number of FB-positive mitral cells

were observed, and the number of FB-positive cells appeared to be proportional to the number of residual fibers in the injured LOT. In these incompletely LOT-transected cases, the BDA-positive bulbar fibers and terminals extended to the posterior one-third of the olfactory cortex and comprised regenerated and residual uninjured axonal elements; this distribution was similar to the distribution observed in normal rats. Therefore, the present study successfully mapped the re-innervated cortical areas of the spontaneously regenerated olfactory structures. It was not clear why these regenerated fibers did not extend to the posterior olfactory cortex. It is well established that a loss of sensory receptor input can lead to organizational changes in the brain [8,9,10,20,21] and it is possible that similar changes arise in the posterior olfactory cortex of neonatally LOT-transected rats. We showed that incomplete regeneration in terms of the cortical areas was sufficient to restore olfactory function, as demonstrated by the olfactory discriminative ability of completely LOT-transected rats in a previous study [15].

We consistently found that neonatally LOT-transected rats demonstrated newly-formed myelinated fiber bundles (ectopic LOTs) near the rhinal sulcus. These results suggest that ectopic myelination occurred after neonatal LOT injuries, irrespective of the lesion sizes. Myelination of LOT occurs between P7 and P21 [13,16]; LOT that was injured at the early neonatal stage comprised unmyelinated fiber bundles. Therefore, post-injury axonal regeneration and postnatal myelination occurred concomitantly. We could not explain why myelination occurred at the lateral edge near the rhinal sulcus in the completely LOT-transected rats instead of the original LOT region or why myelination occurred additionally near the rhinal sulcus despite the persistence of the original LOT in the incompletely LOT-transected rats. However, the positive and negative regulators of myelination [3,11,12,22,23] may have different

expression patterns near the rhinal sulcus in neonatally LOT-transected rats. Furthermore, it would be interesting to determine the proportion of BDA-positive cortical terminals that are derived from ectopic myelinated fiber bundles. Lesion experiments could be performed to establish the functional significance of these ectopic LOTs and to determine whether transection of ectopic myelinated fiber bundles induces olfactory disturbances in completely LOT-transected rats.

In the further experiments using different developmental stages of rats whose LOTs were transected on P7, P14, and P21, mitral cell labeling by a retrograde fluorescent tracer that was injected into the posterior olfactory cortex 5 weeks after LOT transection occurred only in rats with its transection on P7, indicating that the critical period of spontaneous regeneration of the LOT was between 7 and 14 postnatal days (data not shown). Different glial reactions were found in the LOTs transected on P7 and P14 with mild astrocytic reaction of the former and its intense reaction of the latter, and astrocytes at the injured site were known to produce axon-inhibitory molecules [4,6,19]. Similar critical periods of axonal regeneration in the corticospinal tract of different developmental stages of rats who received spinal cord injuries on P0, P6, and P12 were reported to be between 6 and 12 postnatal days [2,5].

In conclusion, the regenerated bulbar fibers extended to specific regions of the anterior olfactory cortices in neonatally LOT-transected rats, whereas ectopic myelination of the regenerated LOT occurred only at one specific site near the rhinal sulcus in completely and incompletely LOT-transected rats.

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Legends for Figures

Fig. 1. (A) Ventral view of a completely LOT-transected case. The left transected LOT cannot be seen as a white myelinated band by lack of myelination. Note the newly-formed myelinated fiber bundle (arrows) on the left transected side. An arrowhead points to the right uninjured LOT. (B) An injection site of FB into the left posterior olfactory cortex. (C) FB-positive mitral cells are absent in the left olfactory bulb. (D and E) Sections processed for immunohistochemistry using an anti-MBP antibody. Note the newly-formed myelinated fibers on the left transected side (arrows). Arrowheads point to the right uninjured LOT. D and E correspond to d and e in A. (F and G) Injection sites of BDA into the right (F) and left (G) olfactory bulbs. (H–J) BDA-positive bulbar terminal regions. BDA-positive terminal zones (asterisks) cannot be seen at the posterior olfactory cortex on the left transected side. H–J correspond to h–j in A. Scale bars. B and D–J: 1 mm; C: 100 μm.

Fig. 2. (A) Ventral view of an incompletely LOT-transected case with a white narrow band of the LOT. Note the newly-formed myelinated fiber bundle (arrows) on the left transected side. Arrowheads point to the right uninjured and left transected LOTs. (B) An injection site of FB into the left posterior olfactory cortex. (C) FB-positive mitral cells can be seen in the left olfactory bulb. (D and E) Sections processed for immunohistochemistry using an anti-MBP antibody. Note the newly-formed myelinated fibers on the left transected side (arrows). Arrowheads point to the right uninjured and left transected LOTs. D and E correspond to d and e in A. (F and G) Injection sites of BDA into the right (F) and left (G) olfactory bulbs. (H–J) BDA-positive bulbar terminal regions. BDA-positive terminal zones (asterisks) can be seen at the most caudal end of

the posterior olfactory cortex on the left transected side. H–J correspond to h–j in A. Scale bars. B and D–J: 1 mm; C: 100 μm.

Fig. 3. Distributions of BDA-positive bulbar terminals. (A) A completely LOT-transected case. (B) An incompletely LOT-transected case. AON: anterior olfactory nucleus; LOT: lateral olfactory tract; OB: olfactory bulb; OT: olfactory tubercle; PC: piriform cortex. Asterisks indicate the cortical amygdaloid nucleus (**) and entorhinal cortex (***). The model diagrams are adapted with permission from Y. Sekiguchi, N. Fukushima, K. Yokouchi, K. Kawagishi, S. Hirayama, T. Moriizumi, Functional correlation between olfaction and various sectioning of the lateral olfactory tract, Neuroscience research 73 (2012) 17–23.

Fig. 1

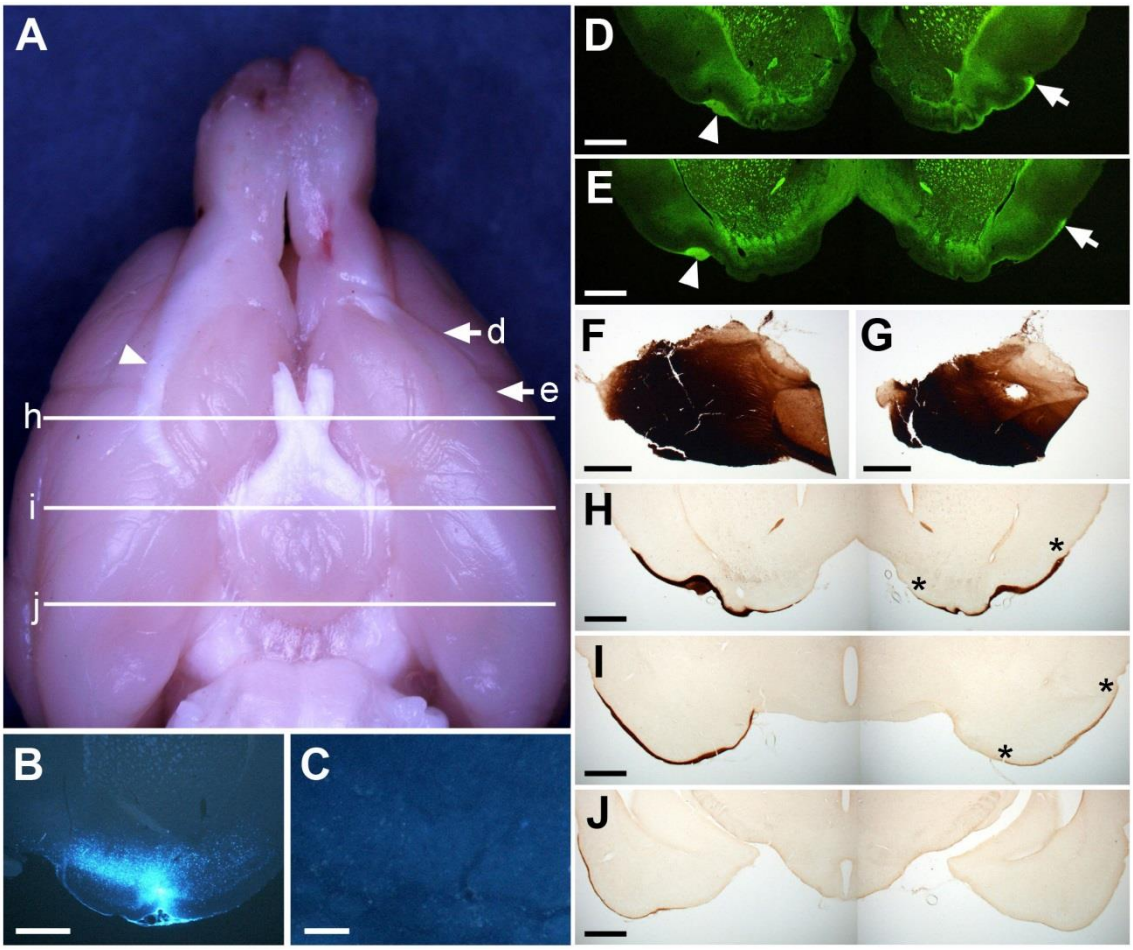

Fig. 2

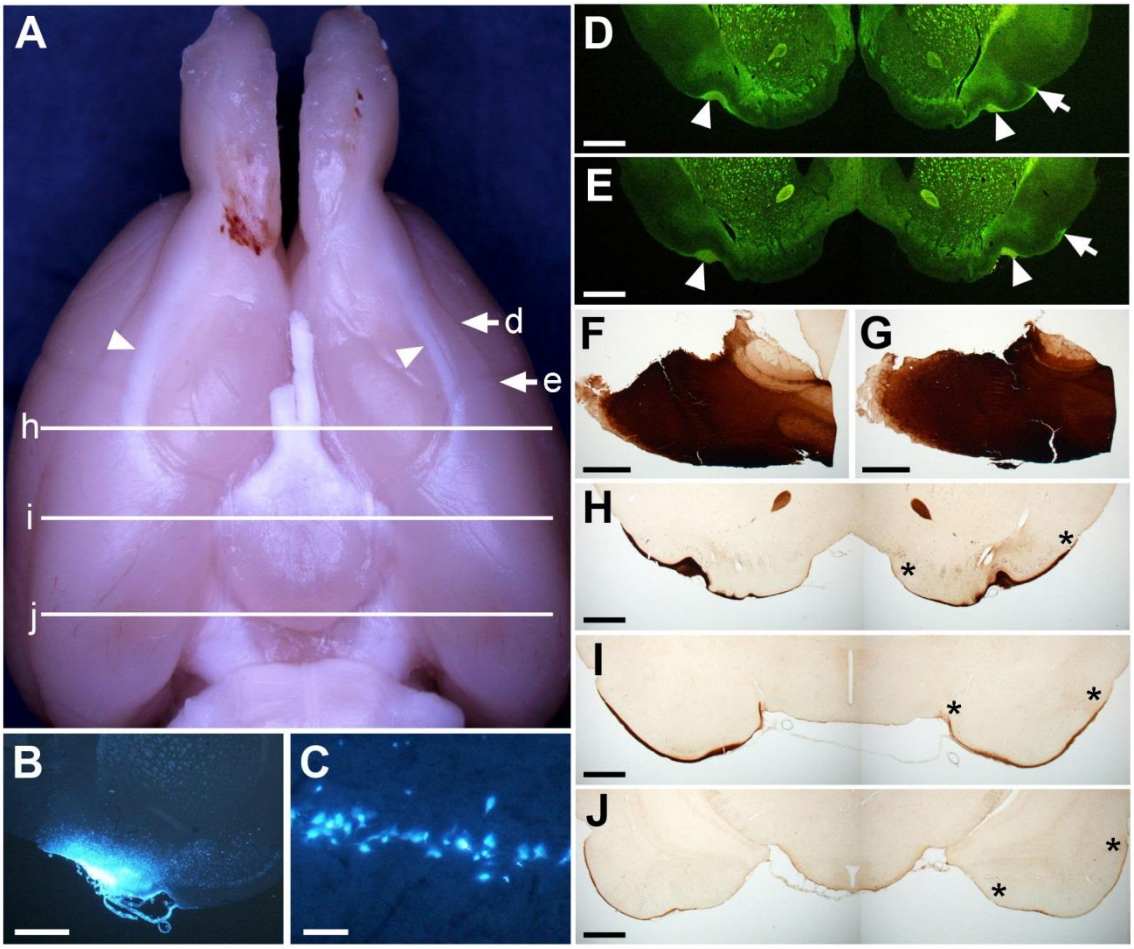

Fig. 3