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Re-evaluation of spontaneous regeneration of the lateral olfactory tract

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ABSTRACT

Spontaneous regeneration of the lateral olfactory tract (LOT) was re-evaluated in newborn rats using a fluorescent retrograde neuronal tracer as objective indicators of complete LOT transection. Complete LOT transection was evidenced by the loss of the white myelinated band characteristic for adult LOT and the total lack of retrograde neuronal labeling of mitral cells by Fast Blue that was injected during LOT transection. In completely LOT-transected young adult rats, mitral cells were retrogradely labeled consistently only by Fluoro-Gold that was injected into the olfactory cortex at the adult stage. Moreover, an anterograde neuronal tracer, biotinylated dextran amine (BDA), was demonstrated to pass from the neonatally LOT-transected bulb, through the transected retrobulbar site, towards the olfactory cortex, far caudally at a level near the optic chiasm. The regenerated structures lacked immunoreactivity for myelin basic protein and electron-dense myelinated axon bundles, and were also characterized by the thinness of the BDA(+) terminal zone within the olfactory cortex and the lack of its caudal extension. Young adult rats subjected to unilateral bulbectomy contralateral to the neonatally LOT-transected side showed perfect ability to discriminate cycloheximide solution by olfaction. From these findings, we conclude that the spontaneously regenerated olfactory system is functional despite structural incompleteness.

1. Introduction

It is accepted that regeneration of the fiber tracts of the central nervous system occur in neonatal or young brains (Devor, 1975; Small and Leonard, 1983; Munirathinam et al., 1997; Inoue et al., 1998; Kikukawa et al., 1998; Ito et al., 1998). Such regeneration is still generally difficult in adult brains and occurs only in certain restricted conditions (Rhodes and Fawcett, 2004; Laabs et al., 2005; Hata et al., 2006; Kaneko et al., 2006; Busch and Silver, 2007; Fu et al., 2007; Lee et al., 2009; Wang et al., 2009). The lateral olfactory tract (LOT), the main fiber tract of the central olfactory system that connects the olfactory bulb to the olfactory cortex, was reported to undergo spontaneous regeneration after 8 months of transection in newborn rats (Munirathinam et al., 1997). However, critical readings of this paper have cast doubt on several important points. First, LOT lesion site was indistinct and it was uncertain whether LOT transections were assessed in a scientific manner (i.e., whether LOT was completely or incompletely transected). Second, LOT regeneration was evaluated only by retrograde neuronal tracing, and not by direct visualization of regenerated axon fibers by anterograde neuronal tracing. Third, LOT regeneration did not occur within 2 months after its neonatal transection, but occurred 8 months later. Whether such a long time is necessary for LOT regeneration needs to be re-examined.

In the present study, LOT transection was accompanied by simultaneous injection of a retrograde neuronal tracer into the bulbar target site (the posterior olfactory cortex) in order to select completely LOT-transected cases that showed no neuronal labeling of the bulbar projection neurons (mitral cells) and to exclude incompletely LOT-transected cases that showed retrograde neuronal labeling of the mitral cells. The present study, which used completely LOT-transected cases, was undertaken to re-evaluate spontaneous LOT regeneration and to investigate the following important issues: (1) demonstration of regenerated mitral cells by retrograde neuronal tracing; (2) whether regenerated axon fibers can be demonstrated by anterograde neuronal tracing; (3) assessment of the macroscopic, microscopic and electron microscopic features of normal and regenerated LOTs; and (4) the olfactory function of the regenerated olfactory nervous system at the adult stage.

2. Materials and methods

2.1. Animals

The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the protocols were

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approved by our Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. Newborn rat pups of the Wistar strain (Japan SLC Inc., Hamamatsu, Japan) of both sexes were used. Postnatal day (P) 0 refers to the first 24 h after birth. Surgical manipulations were performed under general anesthesia with hypothermia (−20 °C, 15 min) on neonatal pups on P1 and P2. Rats at more advanced stages (4 weeks, 6 weeks and 8 weeks) were anesthetized by subcutaneous injection of a mixture of pentobarbital (50 mg/kg) and medetomidine (10 mg/kg). To reverse the anesthesia, antipamezole hydrochloride (2 mg/kg, i.p.) was injected.

2.2. LOT transection combined with injection of a retrograde fluorescent tracer at the neonatal stage

P1–P2 pups ($n = 78$) were subjected to unilateral LOT transection on the left side by inserting the tip of a knife (Ophthalmic Scleral MVR Knife, 25 gauge; Alcon, Tokyo, Japan) from the ventrolateral aspect of the head between the eye and the ear. LOT was transected at the posterior half of the olfactory stria. Immediately after LOT transection, a retrograde fluorescent tracer was injected into the posterior olfactory cortex (the olfactory tubercle and the piriform cortex) situated far caudal to the site of LOT transection. Two fluorescent tracers, Fluoro-Gold (FG) (Fluorochrome, Denver, CO, USA) and Fast Blue (FB) (Polysciences Inc., Warrington, PA, USA), were initially used for retrograde neuronal tracing. A small volume (0.1–0.2 μl) of 2% FG or 1% FB was injected into the posterior olfactory cortex. Our preliminary experiments on neonatal rats showed that FB was more advantageous than FG as a long-term neuronal tracer, because of clear visualization of the injection site of FB and excellent configuration of FB-labeled mitral cells even after 8 weeks of survival. Therefore, LOT transection at the neonatal stage was always accompanied by immediate injection of 0.1 μl of 1% FB into the posterior olfactory cortex. After surgery, the pups were housed with their dam. The dam and her pups were kept in a single cage (26 cm × 42 cm × 18 cm) under standard laboratory conditions with a 12-h light/dark cycle and at room temperature (22 °C). Food and water were supplied ad libitum.

2.3. Retrograde neuronal tracing at the adult stage

LOT-transected, FB-injected neonatal rats were allowed to survive for 4 weeks ($n = 10$), 6 weeks ($n = 10$), or 8 weeks ($n = 10$). They received an injection of 0.4–0.6 μl of 2% FG into the posterior olfactory cortex. Two days (48 h) after FG injection, the rats were deeply anesthetized with sodium pentobarbital (80–100 mg/kg, i.p.) and perfused through the heart with 4% paraformaldehyde (4 weeks 100 ml; 6 weeks 150 ml; 8 weeks 200 ml) in 0.1 M phosphate buffer. The brains were removed, postfixed overnight in the same fixative, and soaked in 30% sucrose for 2 days. The left olfactory bulb was cut into sections 30 μm thick in the sagittal plane serially at 180-μm intervals on a freezing microtome, and the left brain was cut into sections 30 μm thick in the coronal plane serially at 360-μm intervals. The sections were serially mounted on coated slides, coverslipped with glycerol, and observed under a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan). Furthermore, part of the sections were processed for immunohistochemical detection of FG. They were incubated overnight in guinea pig anti-FG antibody (1:4000) (Protos Biotech Corporation, New York, NY, USA) at room temperature. After incubated in biotinylated anti-guinea pig immunoglobulins (1:500) (Dako, Glostrup, Denmark) for 1 h and in Alexa 594-conjugated streptavidine (1:1000) (Molecular Probes, Eugene, OR, USA) for 1 h, these sections were observed under a fluorescence microscope.

2.4. Anterograde neuronal tracing at the adult stage

LOT-transected, FB-injected neonatal rats were allowed to survive for 4 weeks ($n = 8$), 6 weeks ($n = 4$), or 8 weeks ($n = 8$). They were subjected to multiple injections of an anterograde neuronal tracer, biotinylated dextran amine (BDA) (BDA-10000; Molecular Probes, Eugene, OR, USA), into the olfactory bulbs on both sides. BDA injections into 4–6 portions of the olfactory bulb were made such as to cover the whole bulb (0.2 μl × 4–6), and the total volume injected per bulb was 0.8–1.2 μl.

Seven days after BDA injections, the rats were similarly perfused through the heart with 4% paraformaldehyde under deep anesthesia. The brains were removed, postfixed overnight in the same fixative, and soaked in 30% sucrose for 2 days. The bilateral olfactory bulbs were cut into sections 50 μm thick in the sagittal plane serially at 300-μm intervals on a freezing microtome, and the brains were cut into sections 50 μm thick in the coronal plane serially at 600-μm intervals. One set of sections were serially mounted on coated slides, coverslipped with glycerol, and observed under a fluorescence microscope.

Another set of sections were processed for detection of BDA. 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 the avidin–biotin–peroxidase complex (1.5%) and Triton X-100 (0.3%), and were visualized with the Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific Inc., Pierce Biotechnology, Rockford, IL, USA). They were mounted on coated slides, stained with cresyl violet or left unstained, and coverslipped with Entellan New.

Floated sections with blue-black reaction product for BDA were also processed for immunohistochemical detection of myelin basic protein (MBP). Briefly, the sections were incubated overnight in mouse anti-MBP antibody (1:10,000) (Protos Biotech Corporation, New York, NY, USA) at room temperature. After washing, they were incubated in Alexa 488-conjugated donkey anti-mouse secondary antibody (1:1000) (Molecular Probes, Eugene, OR, USA) for 2 h. After washing, they were mounted on coated slides, coverslipped with glycerol, and observed under a fluorescence microscope.

The BDA(+) structures, which were termination sites of the bulbar projection neurons, were further processed for ultrastructural analysis to detect morphological differences between normal and neonatally LOT-transected olfactory cortical centers. Small pieces of LOT and the overlying piriform cortex were dissected out at a level rostral to the optic chiasm. They were postfixed with buffered 2% osmium tetroxide, dehydrated in ethanol, processed with propylene oxide, and embedded in Epon. Ultrathin sections were briefly stained with lead citrate and observed with an electron microscope (JEM-1400; JEOL Ltd., Tokyo, Japan).

2.5. Functional analysis followed by retrograde/anterograde neuronal tracing

LOT-transected, FB-injected neonatal rats were allowed to survive for 4 weeks ($n = 22$), or 6 weeks ($n = 6$). To investigate the functioning of the left olfactory system in rats who underwent neonatal LOT transection on the left side, the right olfactory bulb was completely ablated by aspiration with a 21-gauge needle at the adult stage. Besides the neonatally LOT-transected rats ($n = 28$), young adult rats (4–6-week-old female) were subjected to unilateral ($n = 12$) or bilateral ($n = 5$) bulbectomy, or unilateral LOT transection combined with contralateral bulbectomy ($n = 5$). After deprivation of water for 2 days, these four groups of the experimental rats were submitted to odor aversion behavior tests to examine their olfactory ability to discriminate 0.01% cycloheximide solution from water, as described in our previous reports (Moriizumi et al., 1994; Fukushima et al., 2002). Briefly, two bottles were offered to each rat. One contained 0.01% cycloheximide solution, while another water. Since the taste of cycloheximide solution is so disgusting, the rats remember the smell of the solution when they drink it, and thereafter learn to avoid it by olfaction. When the rat drank water, the response was interpreted as a correct response. When the rat drank cycloheximide solution, the response was regarded as a wrong response. The number of correct responses was divided by the number of total responses in each rat, and the percentages of correct responses were calculated in the four experimental groups. Data are expressed as mean ± S.E.M. Statistical significance of the means was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett test as post hoc analysis. P values less than 0.05 were considered significant.

After the olfactory discrimination test, the neonatally LOT-transected, FB-injected young adult rats received an injection of 0.4 μl of FG ($n = 14$) into the posterior olfactory cortex on the left side or multiple injections of 1.0 μl of BDA ($n = 14$) into the remaining left olfactory bulb. As described previously, brain sections including the left olfactory bulb or the left olfactory cortex were examined for the presence of retrograde or anterograde neuronal tracers.

3. Results

3.1. Regeneration demonstrated with a retrograde neuronal tracer (FG)

The neonatal transected site of the LOT is shown in Fig. 1. LOT transection at the neonatal stage produced both complete and incomplete LOT transection. Complete LOT transection was evi-

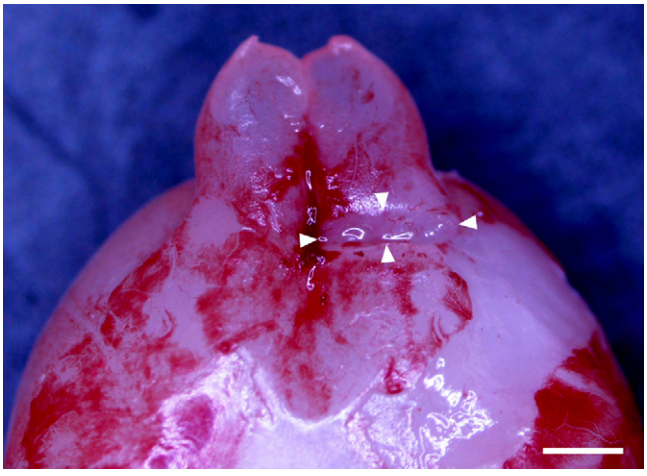

Fig. 1. A ventrobasal aspect of a brain taken from a P2 rat 5 h after transection of the left LOT. Arrowheads point to the transected site. Scale bar: 1 mm.