Morphological analysis of regenerated bulbar fibers in relation to neonatal olfaction

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Abbreviations: BDA, biotinylated dextran amine; FB, Fast Blue; LOT, lateral olfactory tract; P, postnatal day; ROI, region of interest.

ABSTRACT

It was revealed that regeneration of the lateral olfactory tract (LOT) occurred in developing rats and the regenerated olfactory system was functional 4 weeks after transection. The aim of this study was to determine the earliest onset of functional recovery in LOT-injured rats and to quantify regenerated nerve components with functional correlation. Neonatal rats on postnatal day (P) 2 were subjected to unilateral transection of the left LOT and underwent unilateral removal of the right olfactory bulb on P11. Functional recovery of the tract injury was assessed by the suckling capability, which can be achieved by olfaction. Suckling capability was observed on P12 in most neonatally LOT-transected pups. Rat pups were subjected to unilateral transection of the left LOT on P2, and received injections of biotinylated dextran amine (BDA) into the bilateral olfactory bulb on P5 to quantify normal and regenerated nerve components in the olfactory cortices at the level of the olfactory tubercle. BDA(+) areas and density indices of the olfactory cortices in the neonatally LOT-transected P12 pups were 11.05 $\times 10^5$ μm^2 and 0.35 on the normal right side and 4.34 $\times 10^5$ μm^2 and 0.21 on the transected left side. We concluded that functional recovery of the LOT-transected neonatal rats occurred as early as 10 days after tract transection and that areas and densities of regenerated nerve components essential for functional recovery were approximately 40% and 60% of the age-matched normal values in the olfactory cortices at the level of the olfactory tubercle.

Key words: olfactory bulb, lateral olfactory tract, olfactory cortex, tract injury, olfaction

1. Introduction

It is well accepted that neonatal and young animals exhibit regeneration in the fiber tracts of the central nervous system (Devor 1975; Small and Leonard 1983; Munirathinam et al. 1997; Inoue et al. 1998; Ito et al. 1998; Kikukawa et al. 1998; Sherrard and Bower 2001). The lateral olfactory tract (LOT) is the main fiber tract of the central olfactory system and connects the olfactory bulb to the olfactory cortex (the olfactory tubercle and the piriform cortex). We have recently reported that spontaneous regeneration of the LOT consistently occurred in newborn rats and that the regenerated olfactory system was functional 4 weeks after transection (Sakamoto et al. 2010), even when regenerated olfactory structures were incomplete in terms of myelination of the LOT and regenerated cortical areas (Fukushima et al. 2013). Further, it was revealed that such regeneration of the LOT had critical periods early in life and that the proportions of regenerated bulbar projection neurons (mitral cells) gradually decreased during the postnatal two weeks (Hirayama et al. 2014).

The present study was conducted to investigate 2 unsolved issues on the regeneration of the LOT: (1) the earliest onset of functional recovery of the transected LOT and (2) the quantification of regenerated nerve components with functional correlation. Onset of functional recovery was assessed based on the day of acquisition of olfaction that is essential for nipple attachment and subsequent suckling behavior (Bruno et al. 1980; Larson and Stein 1984; Distel and Hudson 1985; Yokouchi et al. 2007; Kawagishi et al. 2009). Regenerated nerve components were visualized using an anterograde neuronal tracer that was injected into the tract-transected olfactory bulb. We will provide quantitative data regarding regenerated bulb-derived nerve components essential for functional recovery.

2. Materials and methods

2.1. Animals

The experiments were performed according to 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 the number of animals used and their suffering. Newborn Wistar rat pups (Japan SLC Inc., Hamamatsu, Japan) of both sexes were used in the study. Postnatal day (P) 0 refers to the first 24 h after birth. Surgical manipulations on the early (P2–P5) and late (P7–P11) neonatal pups were performed in a hypothermic condition using a freezer (−20°C, 15–25 min).

2.2. LOT transection and retrograde tracer injection

LOT transection was performed in P2 pups unilaterally on the left side, as described previously (Sakamoto et al. 2010; Fukushima et al. 2013; Hirayama et al. 2014). Briefly, LOT was transected at the posterior half of the olfactory stria by inserting the tip of a knife (Ophthalmic Scleral MVR Knife, 25 gauge; Alcon, Tokyo, Japan) from the ventrolateral aspect of the head. Immediately after LOT transection, a retrograde fluorescent tracer, Fast Blue (FB) (Polysciences Inc., Warrington, PA, USA), was injected into the left olfactory cortex to confirm the completeness of LOT transection (Sakamoto et al. 2010; Fukushima et al. 2013; Hirayama et al. 2014). FB (1%, 0.1 μl) was injected into the posterior part of the olfactory cortex situated far caudal to the site of LOT transection. After surgery, the pups were housed with their dam and were placed in a single cage (26 × 42 × 18 cm) under standard laboratory conditions with a

12-h light/dark cycle and room temperature of 22°C. Food and water were supplied *ad libitum*.

2.3. Onset of functional recovery in LOT-transected pups

To determine the functioning of the left olfactory system in rats that underwent neonatal LOT transection on the left side, the right olfactory bulb was ablated by aspiration with a 21-gauge needle at the later stages, on P7, P9, and P11. The pups were observed 24 h after unilateral bulbectomy on P8, P10, and P12 if they had milk in the stomach. Stomach milk was easily recognized with the naked eye as whitish substance in the upper abdomen (Fukushima et al. 2006). Stomach milk was observed in part of these pups (P8: $n = 2$; P10: $n = 2$; P12: $n = 4$), whereas no stomach milk was observed in the other pups (P8: $n = 6$; P10: $n = 6$; P12: $n = 4$). By the later histological examination, 6 pups with stomach milk (P8: $n = 2$; P10: $n = 2$; P12: $n = 2$) contained a significant number of FB (+) mitral cells in the left olfactory bulb and were regarded as incomplete LOT-transected cases. These preliminary experiments showed that part ($n = 2$) of the complete LOT-transected P12 pups ($n = 6$) had suckling capability as evidenced by stomach milk.

To investigate the accurate day of functional recovery in the neonatally LOT-transected pups, they were separated from their dam 2–4 hours prior to bulbectomy in order to empty the stomach. A total of 22 P2 pups underwent LOT transection unilaterally on the left side, and the right olfactory bulb was ablated on P11. Suckling capability of these pups was examined as follows. Stomach milk was completely lost 2–4 h after unilateral bulbectomy (P11, 9:00–10:00) by maternal deprivation (Fujita et al. 2006; Fukushima et al. 2006; Fukuyama et al. 2006), which

enabled us to know the approximate time of occurrence of suckling capability. Stomach milk was checked more frequently after bulbectomy on P11 at 14:00, 17:00, 20:00, and 23:00, on P12 at 8:00, 11:00, 14:00, 17:00, 20:00, and 23:00, and on P13 at 8:00, 11:00, 14:00, 17:00, 20:00, and 23:00 (Fig. 1A). Pups without stomach milk were subcutaneously injected with acetated Ringer's solution containing 5% glucose (50 mL/kg) at 12-h intervals (8:00 and 20:00) to increase their survival rates (Fukushima et al. 2007). In addition to the neonatally LOT-transected P11 pups (n = 22), a total of 15 P11 pups received unilateral (n = 10) or bilateral (n = 5) bulbectomy for comparison. The unilaterally bulbectomized pups were served as controls. After the functional test of suckling, the pups were deeply anesthetized with sodium pentobarbital (80–100 mg/kg, i.p.) and perfused through the heart with 4% paraformaldehyde (30–50 ml) in 0.1 M phosphate buffer. The brains were removed, postfixed overnight in the same fixative, soaked in 30% sucrose for 2 days, and cut into frozen sections, as described later in more detail. The sections were observed under a fluorescent microscope to examine FB (+) mitral cells, and incomplete LOT-transected cases were excluded from the experiments.

2.4. Anterograde neuronal tracing of regenerated nerve fibers

LOT transection was performed in P2 pups unilaterally on the left side, and FB was injected into the left posterior olfactory cortex, as described previously. To visualize regenerated nerve fibers from the tract-transected bulb, the neonatally LOT-transected pups received two injections (0.2 μl × 2) of an anterograde neuronal tracer, biotinylated dextran amine (BDA) (BDA-10000; 10%; Molecular Probes, Eugene, OR, USA) into the left bulb on P5. BDA injections (0.2 μl × 2) were also made into the normal right

bulb to trace bulb-derived nerve fibers towards the olfactory cortex.

These LOT-transected, BDA-injected pups (n = 17) were similarly perfused through the heart with 4% paraformaldehyde on P10 (n = 8) and P12 (n = 9), because our preliminary experiments showed that neonatally LOT-transected P10 and P12 pups were at different functional stages in terms of suckling capability. The brains were removed, postfixed overnight in the same fixative, soaked in 30% sucrose for 2 days, and divided into 2 regions (olfactory bulbs and other brain regions). Using a freezing microtome, 50-μm sagittal sections of the olfactory bulb were cut at 300-μm intervals (6 sets). Other brains were cut into a series of 50-μm coronal sections at 300-μm intervals (6 sets). The second set of sections was mounted as a series on coated slides, cover-slipped with glycerol, and observed under a fluorescence microscope to exclude incomplete LOT-transected cases with FB (+) bulbar mitral cells.

The first set of sagittal bulbar sections, and the first, third, and fifth sets of coronal brain 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 in 0.1 M phosphate-buffered saline containing an avidin biotin peroxidase complex (1.5%) and Triton X-100 (0.3%) for 2 h and were visualized with a metal enhanced DAB Substrate Kit (Thermo Fisher Scientific Inc., Pierce Biotechnology, Rockford, IL, USA). Sections were rinsed, mounted on coated slides, air-dried, dehydrated, and cover-slipped with Entellan New (Merck, Darmstadt, Germany) (Fig. 1B).

2.5. Quantitative assessment of areas and densities of regenerated nerve fibers

Morphometric analyses of BDA (+) bulbar projecting fibers were performed using the ImageJ software (Jensen 2013). First, BDA (+) fibers and terminals in the olfactory

cortex (olfactory tubercle and piriform cortex) were captured at the four tubercular levels using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). Based on serial sections obtained at 100-μm intervals, the rostral tip and caudal end of the olfactory tubercle were regarded as levels 1 and 4, and the rostral and caudal one-thirds of the olfactory tubercle were regarded as levels 2 and 3. To view the images of thick samples, Z-stack images were captured at 2.5-μm intervals. We created joint images with the microscope that can combine high-resolution images into a single wide-field image. Second, areas in which BDA (+) fibers and terminals were observed, regarded as BDA (+) areas, were quantified using the Image/Adjust/Threshold/Create Selection tools of the ImageJ 1.48v software. Briefly, the image was first converted into a gray scale image. The threshold was then adjusted until only the BDA (+) fibers and terminals were selected by the Cut tool. The region of interest (ROI) was defined by lining the border of the selected region with the Create Selection tool and the area of ROI could be measured by the ROI manager tool. BDA (+) areas were quantified at the four levels (1–4) of the olfactory tubercle on both sides (left, LOT-transected; right, normal) in P10 and P12 pups with complete transection of LOT. Similarly, quantification of BDA (+) densities immediately above LOT was performed in P10 and P12 pups. BDA (+) density, expressed as BDA (+) density index, was regarded as the proportion of the occupied area of the BDA (+) fibers and terminals in the rectangular area of 1.0×10^4 μm^2. The data were expressed as mean ± standard deviation (SD). Statistical significance of the means was evaluated by Student's *t*-test. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. Functional recovery after LOT transection

Maternal deprivation resulted in the total absence of stomach milk in all experimental pups at the first observation time (14:00) on P11 just after unilateral or bilateral bulbectomy. In control pups (n = 10) with only unilateral bulbectomy, stomach milk was observed on P11 and thereafter. However, no stomach milk was observed on P11, P12, or P13 in the bilaterally bulbectomized pups (n = 5). In contrast, time of appearance of milk in the stomach very differed among the neonatally LOT-transected pups (n = 22). Stomach milk was observed on P11 and thereafter in 6 pups, on P12 and thereafter in 10 pups, and on P13 in 4 pups. Stomach milk was not observed on P11, P12, or P13 in 2 pups. Postmortem macroscopic views of such pups showed involvement of injuries to left bulbs; one bulb was heavily covered with blood clots and another bulb was very atrophic.

The histological examination revealed that 7 pups with neonatal LOT transection contained a number of FB (+) mitral cells in the left olfactory bulb and were regarded as incomplete LOT-transected cases; 6 pups with appearance of milk in the stomach on P11 and one pup with appearance of milk in the stomach on P12. In summary, among the complete LOT-transected pups, stomach milk was not observed on P11, but first observed on P12 (n = 9) or P13 (n = 4) (Fig. 2). Fig. 3 shows a macroscopic view of a representative case with unilateral transection of the left LOT on P2 and unilateral removal of the right olfactory bulb on P11.

3.2. Morphological analysis of regenerated nerve fibers

As described previously, we excluded the incomplete LOT-transected pups who contained a substantial number of FB (+) mitral cells in the bulb. In such pups, BDA (+)

fibers and terminals extended beyond the olfactory cortex at the level of the olfactory tubercle to the more posterior piriform cortex, which was similar to those fibers in normal pups. Fig. 4A shows a representative case of a P10 pup that underwent unilateral transection of the left LOT on P2 and bilateral injections of BDA into the bulb on P5. BDA (+) fibers and terminals from the tract-transected left bulb were observed in the olfactory cortex at the three rostral levels (1, 2, and 3) of the olfactory tubercle (Fig. 4B) in P10 pups (n = 5). The olfactory cortex at the caudal tubercular level (4) lacked BDA (+) fibers and terminals. BDA (+) areas were much smaller in the tract-transected left cortices (level 1, 1.07 ± 0.39 × 10^5 μm^2; level 2, 0.56 ± 0.29 × 10^5 μm^2; level 3, 0.29 ± 0.31 × 10^5 μm^2; level 4, 0 μm^2; total, 1.91 ± 0.93 × 10^5 μm^2) than those in the normal right cortices (level 1, 2.39 ± 0.68 × 10^5 μm^2; level 2, 2.31 ± 0.69 × 10^5 μm^2; level 3, 2.14 ± 0.48 × 10^5 μm^2; level 4, 1.81 ± 0.32 × 10^5 μm^2; total, 8.66 ± 1.89 × 10^5 μm^2) at each tubercular level and in total (Table 1) (Fig. 4B, C, Fig. 5A, B). The percentages of BDA (+) areas of the tract-transected cortices relative to the normal cortices were 44% ± 11%, 23% ± 7%, 12% ± 12%, and 0% at each tubercular level and 21% ± 6% in total (Fig. 5D, E).

Fig. 6A shows a representative case of a P12 pup that underwent unilateral transection of the left LOT on P2 and bilateral injections of BDA into the bulb on P5. BDA (+) fibers and terminals from the tract-transected left bulb were observed in the olfactory cortex at all four levels of the olfactory tubercle in two of six P12 pups (Fig. 6B). However, the olfactory cortex at the caudal tubercular level (4) lacked BDA (+) nerve components in four pups. Compared with BDA (+) areas in the tract-transected left cortices in P10 pups, BDA (+) areas in P12 pups showed increased values at all four tubercular levels (level 1, 2.38 ± 0.53 × 10^5 μm^2; level 2, 1.29 ± 0.47 × 10^5 μm^2; level 3,

0.65 ± 0.57 × 10^5 μm^2; level 4, 0.02 ± 0.04 × 10^5 μm^2) and in total (4.34 ± 1.42 × 10^5 μm^2) (Table 1) (Fig. 5A, C, Fig. 6B, C). Corresponding values in the normal right cortices in P12 pups were 3.40 ± 0.66 × 10^5 μm^2, 2.99 ± 0.30 × 10^5 μm^2, 2.53 ± 0.36 × 10^5 μm^2, and 2.14 ± 0.44 × 10^5 μm^2 at tubercular levels 1, 2, 3, and 4, respectively, and 11.05 ± 1.18 × 10^5 μm^2 in total (Table 1) (Fig. 5A, C, Fig. 6B, C). The percentages of BDA (+) areas of the tract-transected cortices relative to the normal cortices were 75% ± 26%, 45% ± 16%, 31% ± 22%, and 1% ± 1% at tubercular levels 1, 2, 3, and 4, respectively, and 41% ± 12% in total (Fig. 5D, E).

BDA (+) density indices in the tract-transected left cortices near the LOT showed higher values in P12 pups (level 1, 0.37 ± 0.09; level 2, 0.28 ± 0.09; level 3, 0.15 ± 0.11; level 4, 0.04 ± 0.06) than those indices in P10 pups (level 1, 0.21 ± 0.10; level 2, 0.22 ± 0.07; level 3, 0.10 ± 0.10; level 4, 0) at all four levels of the olfactory tubercle (Table 1) (Fig. 4D, E, Fig. 6D, E, Fig. 7A, B). The percentages of BDA (+) densities of the tract-transected cortices relative to the normal cortices at each tubercular level were 66% ± 22%, 62% ± 15%, 25% ± 22%, and 0% at levels 1, 2, 3, and 4, respectively, in P10 pups, and 131% ± 36%, 79% ± 35%, 47% ± 39%, and 9% ± 13% at levels 1, 2, 3, and 4, respectively, in P12 pups (Fig. 7C).

4. Discussion

Regeneration of the central nervous system was reported to occur in the pyramidal tract (Inoue et al. 1998), central auditory pathway (Ito et al. 1998), dorsal column projection (Kikukawa et al. 1998) and olivocerebellar projection (Sherrard and Bower 2001) in developing animals. The LOT was also reported to undergo regeneration after 8 months of transection in neonatal rats using a retrograde neuronal tracer

(Munirathinam et al. 1997). However, our recent study (Sakamoto et al. 2010) has demonstrated that regeneration of the neonatal LOT occurred 4 weeks after transection using anterograde and retrograde neuronal tracers. It is important to consider the functional ability of the spontaneously regenerated fiber tracts, but few studies are available regarding the earliest onset of occurrence of functional recovery by regenerated fibers in the central nervous system. Male neonatal hamsters with LOT transection were demonstrated to show mating behavior at the adult stage (Devor 1975). Small and Leonard (1983) reported that neonatal hamsters with LOT transection showed functional recovery 10 days after transection by the phenomenon of persistent thermotaxis. Furthermore, goldfish with unknown age were shown to acquire the ability to discriminate stimulus concentration differences 2 weeks after LOT transection (von Rekowski and Zippel 1993). Recently, we have reported that neonatal rats with LOT transection had olfactory discriminative ability 4 weeks after transection (Sakamoto et al. 2010), and thus it is interesting to determine how soon functional recovery occurs after transection. In this connection, olfactory function of developing rats was shown to be examined by their suckling capability (Yokouchi et al. 2007; Kawagishi et al. 2009), because rat pups use olfactory cues for nipple attachment during the suckling period. Therefore, the exact time of functional recovery of the transected LOT awaited to be determined.

The accurate day of functional recovery in the neonatally (P2) LOT-transected pups was determined by the day of acquisition of suckling capability that can be achieved by olfaction. Although the control pups with unilateral bulbectomy showed suckling capability on P11 (100%: 10/10), suckling capability did not occur on P11 in the neonatally LOT-transected pups. Suckling capability of the neonatally LOT-transected

pups occurred on P12 (69%: 9/13) or P13 (31%: 4/13). Our preliminary experiments showed that suckling capability occurred on P12 in 33% (2/6) of the neonatally LOT-transected pups. The relatively higher percentage (69%) of occurrence of suckling capability on P12 in the present experiments is likely associated with frequent observations of stomach milk daily between 8:00 and 23:00 and to subcutaneous injections of acetated Ringer's solution with 5% glucose in pups without stomach milk until they were capable of suckling. We concluded that functional recovery of the neonatally LOT-transected pups occurred as early as 10 days after tract transection.

To our knowledge, no data are available regarding the quantification of regenerated nerve components essential for functional recovery. We employed BDA, an anterograde neuronal tracer, for the visualization of regenerated bulbar fibers. Anterograde axonal transport of BDA usually requires survival times ranging from several days to 1–2 weeks based on the nervous system of different animals. In the present study, intrabulbar injections of BDA were made on P5 in the neonatally (P2) LOT-transected pups, and thus BDA was injected during axonal regeneration. BDA was successfully traced throughout the long distance from the tract-transected left bulb to the left olfactory cortex at the level of the olfactory tubercle, the caudal structure far from the LOT-transected site. The LOT-transected, BDA-injected pups were sacrificed on P10 and P12 to quantify the amounts of regenerated nerve components in the olfactory cortex.

BDA (+) areas and densities of regenerated nerve components in neonatal pups were compared before functional recovery on P10 and after functional recovery on P12. BDA (+) areas of regenerated nerve components in the olfactory cortices increased 2.3 times between P10 ($1.91 \pm 0.93 \times 10^5$ μm^2) and P12 ($4.34 \pm 1.42 \times 10^5$ μm^2). Percentages of

these BDA (+) areas relative to age-matched normal values increased 2.0 times between P10 (21%) and P12 (41%). BDA (+) density indices of regenerated nerve components in the olfactory cortices also increased 1.6 times between P10 (average value, 0.13) and P12 (average value, 0.21). In particular, density indices in P12 pups greatly increased in the olfactory cortices at the two rostral tubercular levels (level 1, 0.37; level 2, 0.28). Because BDA (+) density indices in the corresponding olfactory cortices on the normal right side were 0.34 (average value) in P10 pups and 0.35 (average value) in P12 pups, BDA (+) density indices of regenerated nerve components relative to age-matched normal values increased 1.6 times between P10 (38%: 0.13/0.34) and P12 (60%: 0.21/0.35). We concluded that areas and densities of regenerated nerve components essential for functional recovery were approximately 40% and 60% of the age-matched normal values in the olfactory cortices at the tubercular level.

Because LOT was transected at the caudal half of the olfactory stria, BDA (+) bulbar fibers and terminals in the olfactory cortex at the level of the olfactory tubercle can be regarded to be all regenerated nerve components. It should be mentioned here that no BDA (+) fibers and terminals were confirmed to reach the olfactory cortex at the tubercular level in neonatally LOT-transected P6 pups, indicating no residual bulbar fibers in the complete LOT-transected olfactory cortex on P6 (unpublished data). It is of great interest that regenerated nerve components essential for functional recovery are very small in quantity compared with the very large size of the olfactory cortex widely situated at the ventrobasal aspect of the brain because small unilateral cortical area at the tubercular level is sufficient for olfactory perception resulting in nipple attachment. Furthermore, smaller (41% of the normal value) cortical areas with lower (60% of the normal value) density of regenerated nerve components appear to be functional, as

described above.

In some neonatally (P2) LOT-transected pups, BDA (+) regenerated fibers reached the olfactory cortex at the most caudal level of the olfactory tubercle on P12. Thus, the leading front of the regenerating fibers is the caudal end of the olfactory tubercle on P12. Because the distance between the caudal half of the olfactory stria (the transected site) and the caudal end of the olfactory tubercle is approximately 3.5–4.5 mm in length, the elongation speed of the fastest components of the regenerating fibers is estimated to be approximately 0.4 mm/day if those fibers elongate continually at the same speed.

In conclusion, functional recovery of the LOT-transected neonatal rats occurred as early as 10 days after tract transection and that areas and densities of regenerated nerve components essential for functional recovery were approximately 40% and 60% of the age-matched normal values in the olfactory cortices at the level of the olfactory tubercle.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Experimental designs of stomach milk observation (A) and BDA detection (B). OB: olfactory bulb.

Fig. 2. Onset of suckling capability in control and LOT-transected pups. After bulbectomy on P11, stomach milk first appeared on P11 (100%) in control pups, while stomach milk first appeared on P11 (86%) or P12 (14%) in incomplete LOT-transected pups, and on P12 (69%) or P13 (31%) in complete LOT-transected pups. Left LOTs were transected on P2, and right olfactory bulbs were removed on P11.

Fig. 3. Macroscopic view of a neonatally (P2) LOT-transected P12 pup. A right olfactory bulb was removed on P11, and stomach milk was observed on P12. An arrow indicates the transected site of the LOT.

Fig. 4. (A) Macroscopic view of a P10 pup. A left LOT was transected on P2, and BDA was injected into both bulbs on P5. (B) BDA (+) bulbar fibers and terminals in the olfactory cortices on the normal side (Right) and the LOT-transected side (Left). (C) Higher magnification of the rectangle in (B). (D) BDA (+) fibers and terminals near LOTs. (E) Higher magnification of the rectangle in (D). The small rectangle in (E) indicates the region that was used for the quantification of BDA (+) density. 1–4 in (B) and (D) correspond to 1–4 in (A). OB: olfactory bulb; OT: olfactory tubercle; PC: piriform cortex.

Fig. 5. (A-C) BDA (+) areas in olfactory cortices on the normal side (right: R) and the

LOT-transected side (left: L) in P10 (B) and P12 (C) pups with unilateral transection of LOTs on P2. BDA (+) areas were measured at the four levels of the olfactory tubercle (B and C) and in total (A). Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ (*versus* normal). (D and E) Percentages of BDA (+) areas of LOT-transected cortices relative to normal cortices at the four levels of the olfactory tubercle and in total. Data are presented as mean ± SD. *$p < 0.05$ (*versus* P10).

Fig. 6. (A) Macroscopic view of a P12 pup. A left LOT was transected on P2, and BDA was injected into both bulbs on P5. (B) BDA (+) bulbar fibers and terminals in olfactory cortices on the normal side (Right) and the LOT-transected side (Left). (C) Higher magnification of the rectangle in (B). (D) BDA (+) fibers and terminals near LOTs. (E) Higher magnification of the rectangle in (D). The small rectangle in (E) indicates the region that was used for the quantification of BDA (+) density. 1–4 in (B) and (D) correspond to 1–4 in (A). OB: olfactory bulb; OT: olfactory tubercle; PC: piriform cortex.

Fig. 7. (A and B) BDA (+) density indices in olfactory cortices on the normal side (right: R) and the LOT-transected side (left: L) in P10 (A) and P12 (B) pups with unilateral transection of LOTs on P2. BDA (+) density indices were measured at the four levels of the olfactory tubercle. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ (*versus* normal). (C) Percentages of BDA (+) density indices of LOT-transected cortices relative to normal cortices at the four levels of the olfactory tubercle. Data are presented as mean ± SD. *$p < 0.05$ (*versus* P10).

Fig. 1

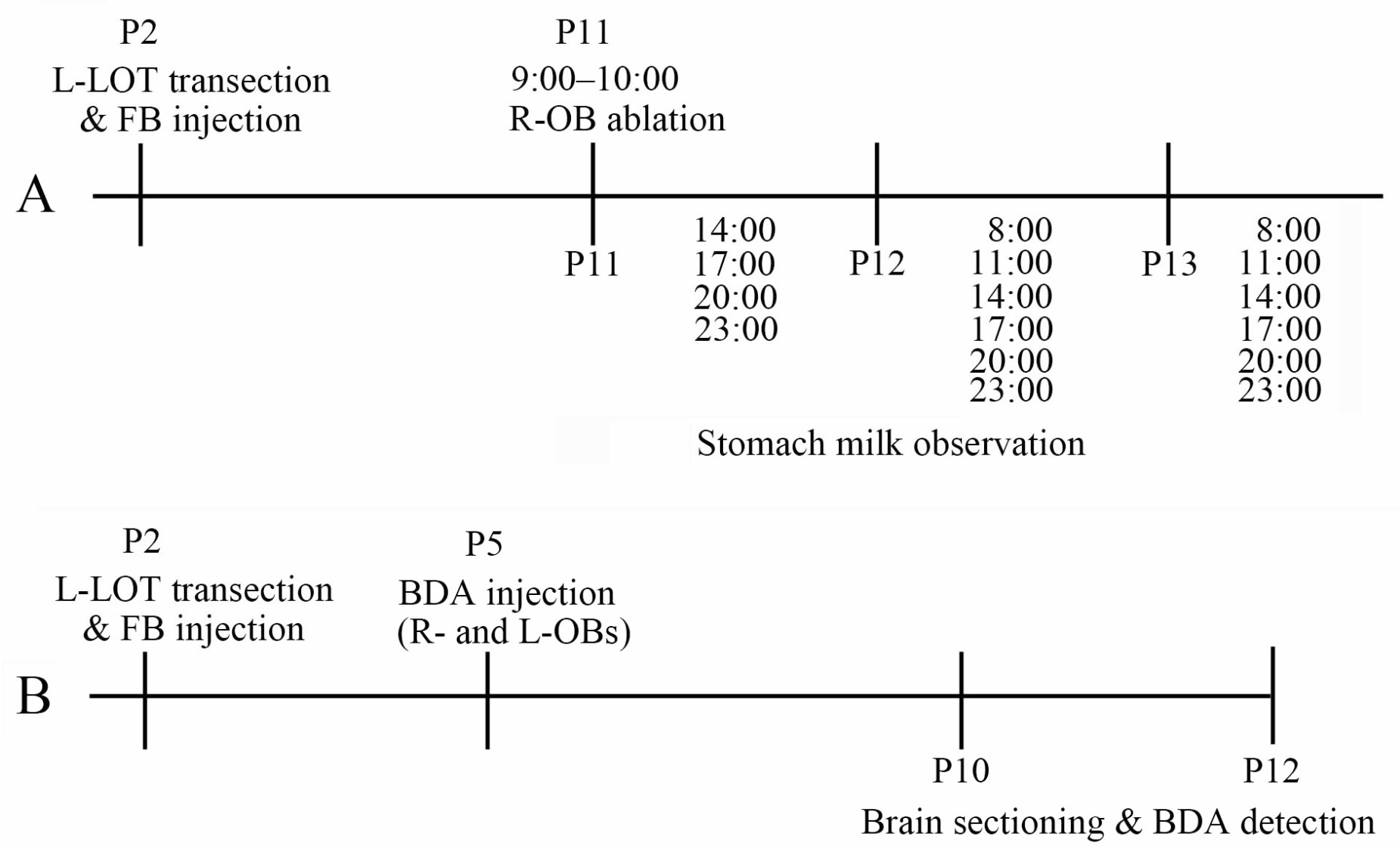
A
P2
L-LOT transection
& FB injection
P11
9:00–10:00
R-OB ablation
P11
14:00
17:00
20:00
23:00
P12
8:00
11:00
14:00
17:00
20:00
23:00
P13
8:00
11:00
14:00
17:00
20:00
23:00
Stomach milk observation
B
P2
L-LOT transection
& FB injection
P5
BDA injection
(R- and L-OBs)
P10
P12
Brain sectioning & BDA detection

Fig. 2

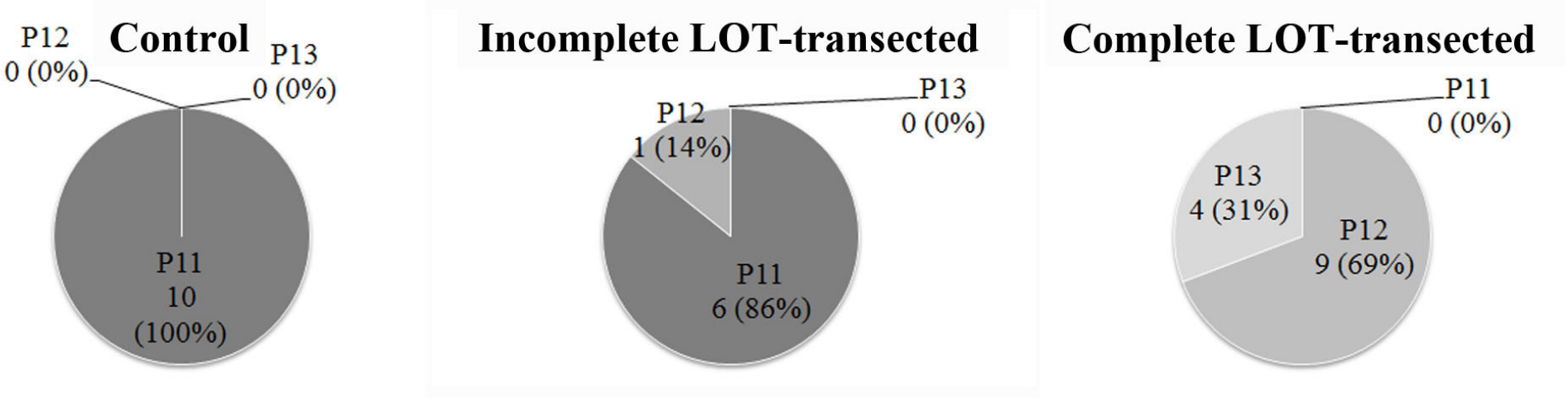
Control
P12
0 (0%)
P13
0 (0%)
P11
10
(100%)
Incomplete LOT-transected
P12
1 (14%)
P13
0 (0%)
P11
6 (86%)
Complete LOT-transected
P11
0 (0%)
P13
4 (31%)
P12
9 (69%)

Fig. 3

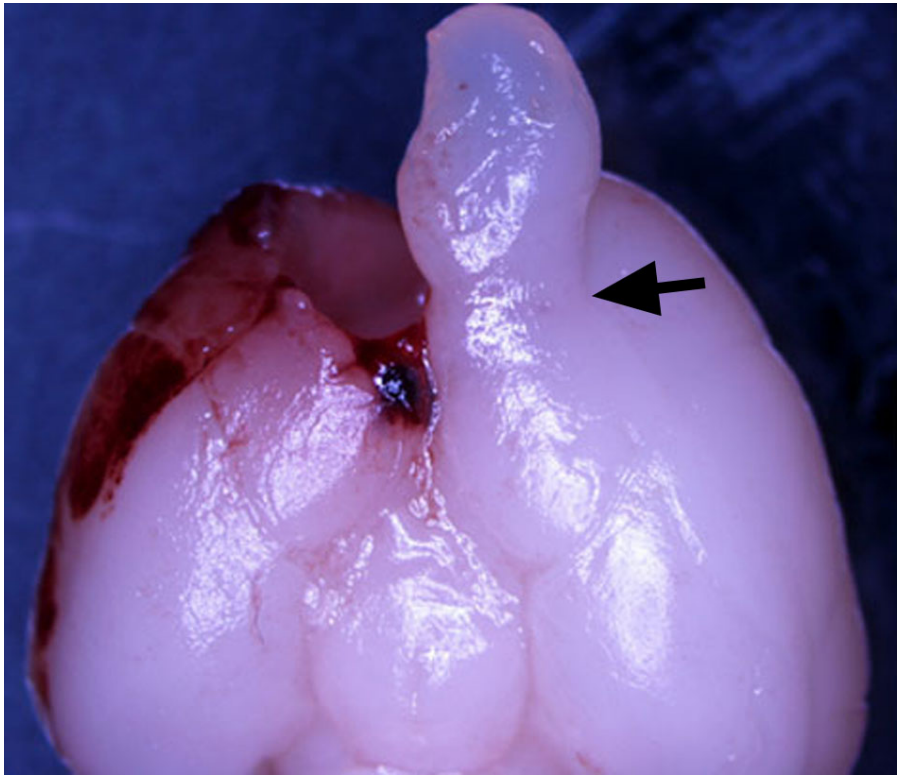

Fig. 4

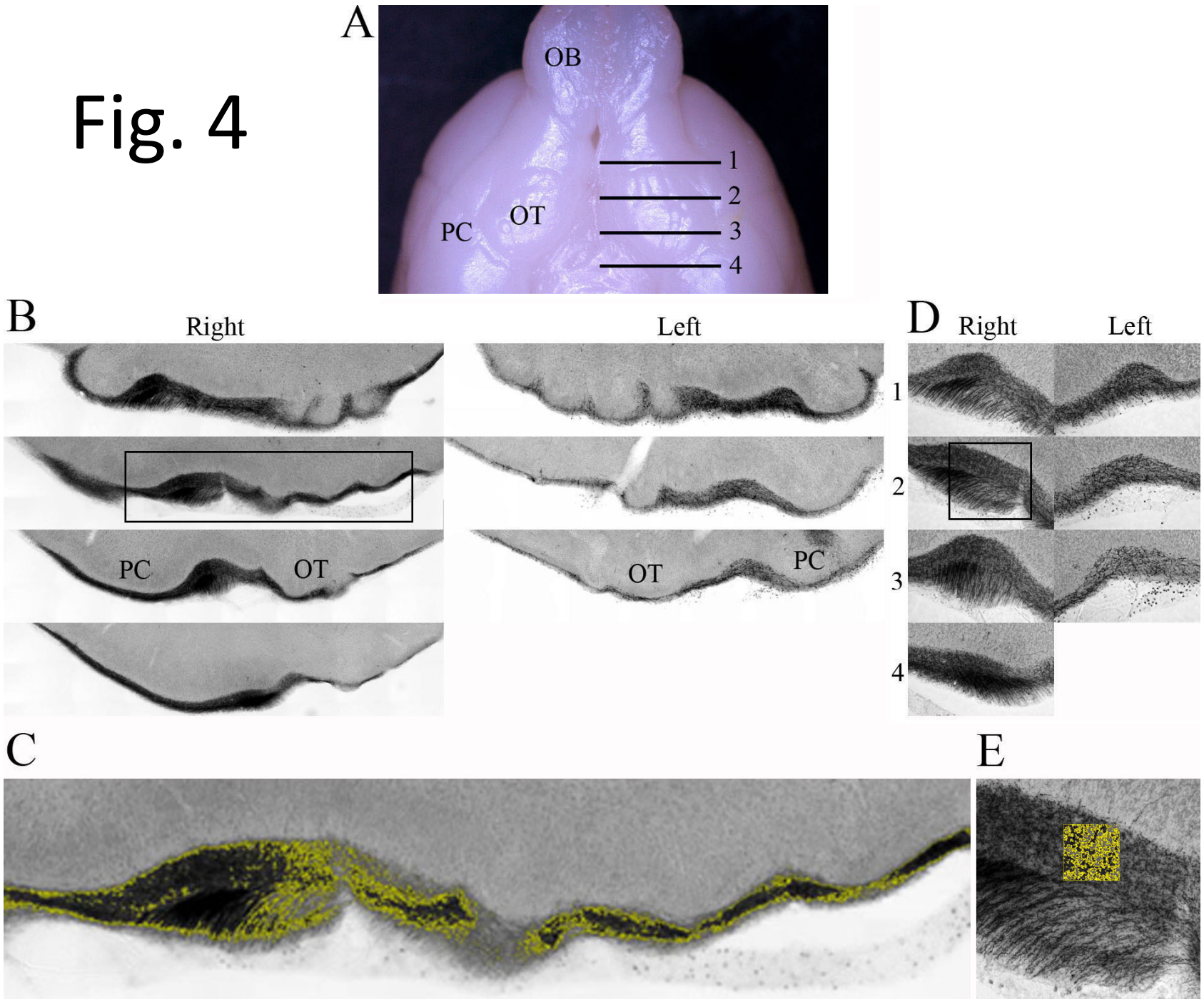

Fig. 5

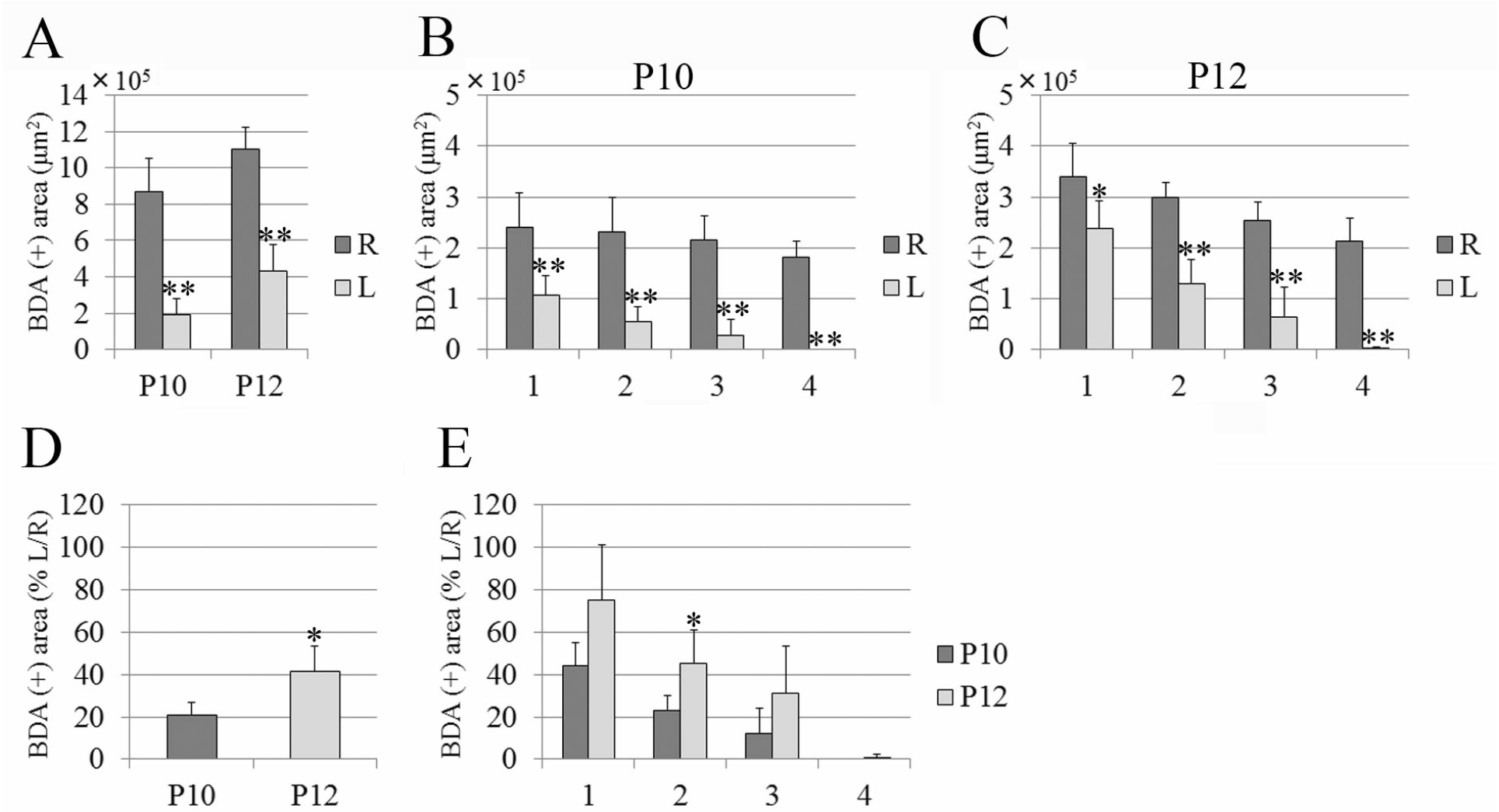

Fig. 6

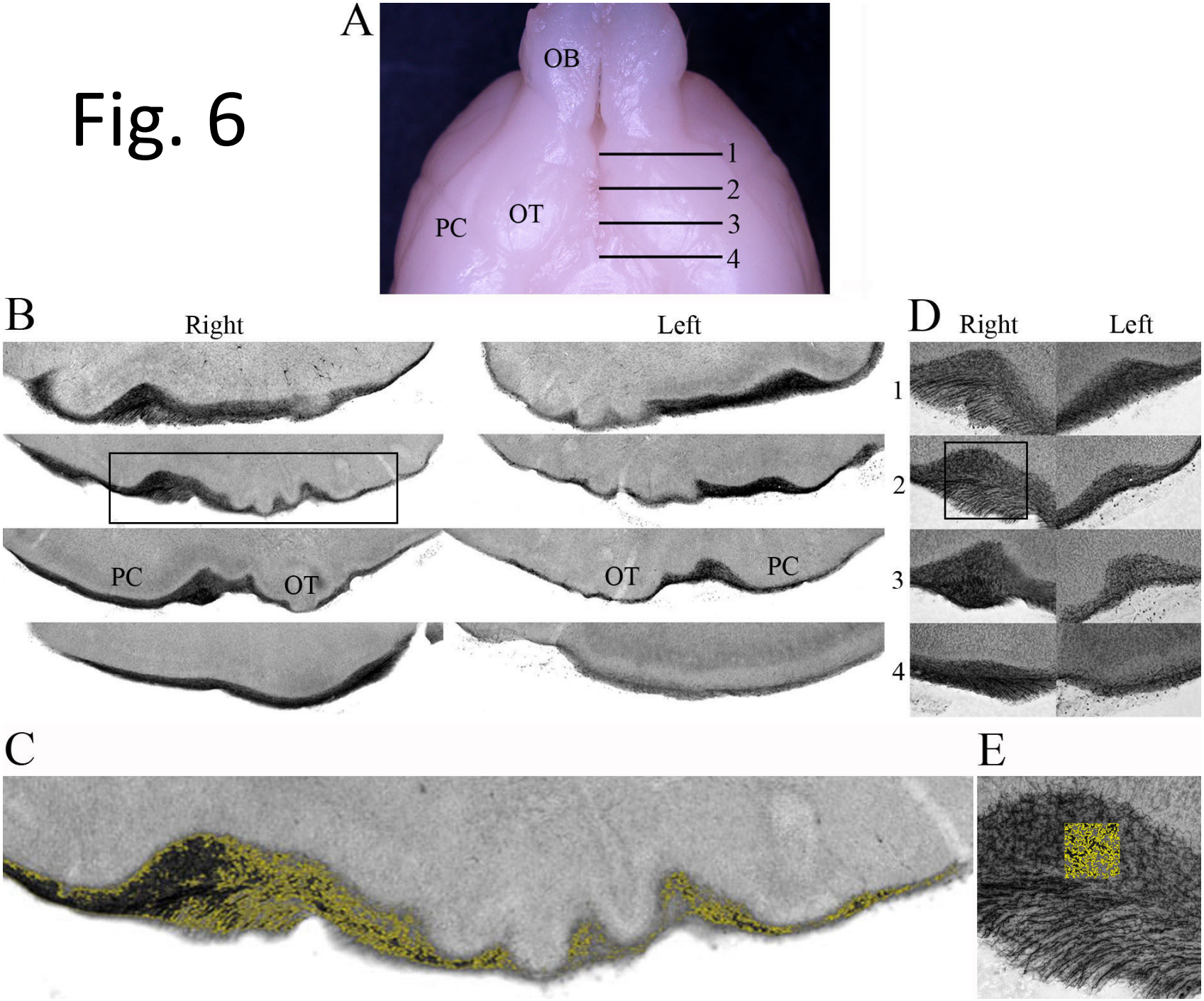

Fig. 7

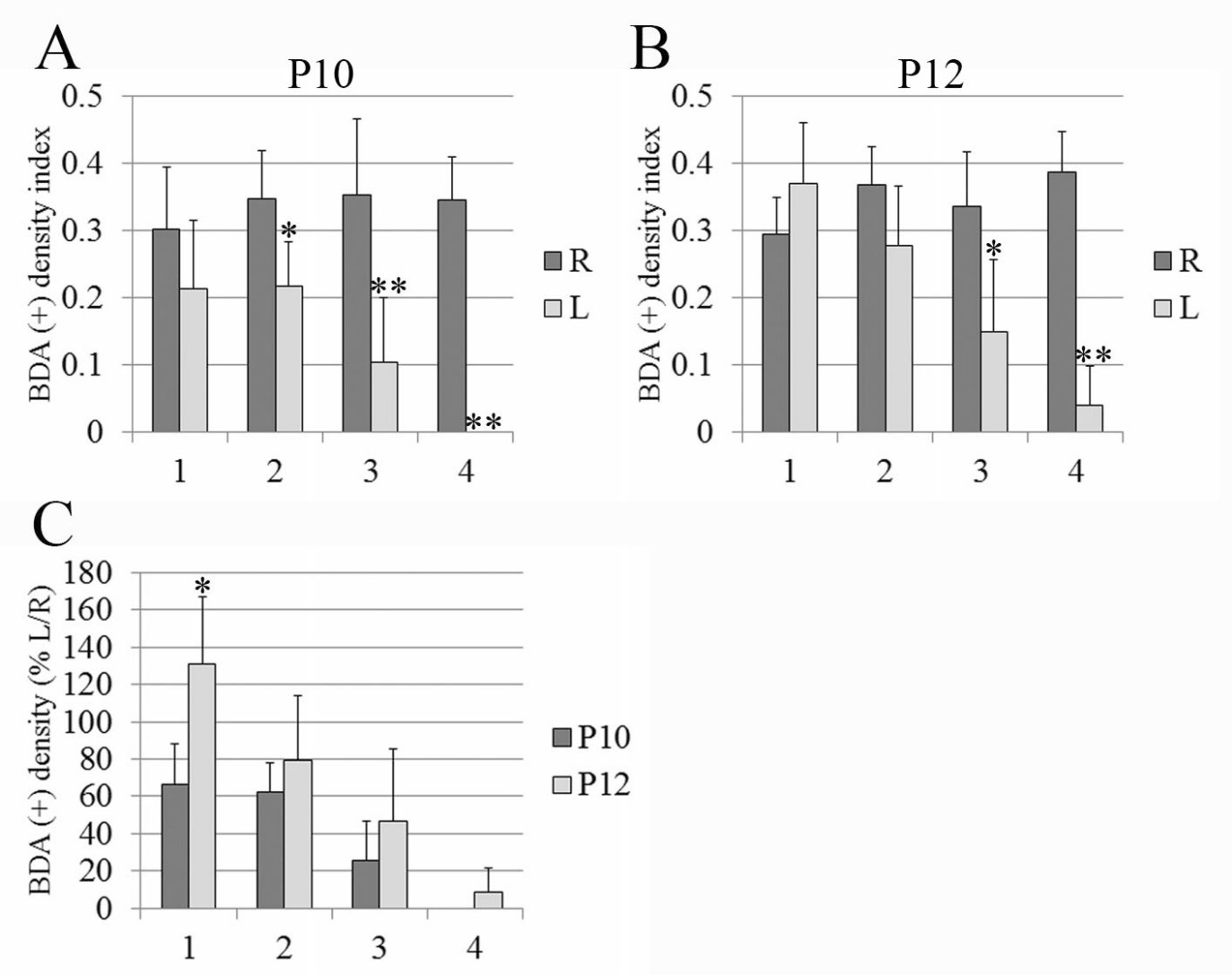
A
P10
BDA (+) density index
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
*
**
**
R
L
B
P12
BDA (+) density index
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
*
**
R
L
C
BDA (+) density (% L/R)
180
160
140
120
100
80
60
40
20
0
1
2
3
4
*
P10
P12

Table 1

Table 1

BDA (+) areas and density indices in P10 and P12 pups.

		BDA (+) areas (× 10^5 μm^2)				BDA (+) density indices			
		P10		P12		P10		P12	
		Right	Left	Right	Left	Right	Left	Right	Left
Level	1	2.39 ± 0.68	1.07 ± 0.39	3.40 ± 0.66	2.38 ± 0.53	0.30 ± 0.09	0.21 ± 0.10	0.29 ± 0.06	0.37 ± 0.09
	2	2.31 ± 0.69	0.56 ± 0.29	2.99 ± 0.30	1.29 ± 0.47	0.35 ± 0.07	0.22 ± 0.07	0.37 ± 0.06	0.28 ± 0.09
	3	2.14 ± 0.48	0.29 ± 0.31	2.53 ± 0.36	0.65 ± 0.57	0.35 ± 0.11	0.10 ± 0.10	0.34 ± 0.08	0.15 ± 0.11
	4	1.82 ± 0.32	0	2.14 ± 0.44	0.02 ± 0.04	0.34 ± 0.07	0	0.39 ± 0.06	0.04 ± 0.06