Brain Research Bulletin 127 (2016) 66–73

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Morphological analysis of regenerated bulbar fibers in relation to neonatal olfaction

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ARTICLE INFO

Article history:
Received 13 June 2016
Received in revised form 4 August 2016
Accepted 25 August 2016
Available online 26 August 2016

Keywords:
Olfactory bulb
Lateral olfactory tract
Olfactory cortex
Tract injury
Olfaction

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 11.05×10^5 μm^2 and 0.35 on the normal right side and 4.34×10^5 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.

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.

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

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http://dx.doi.org/10.1016/j.brainresbull.2016.08.015
0361-9230/

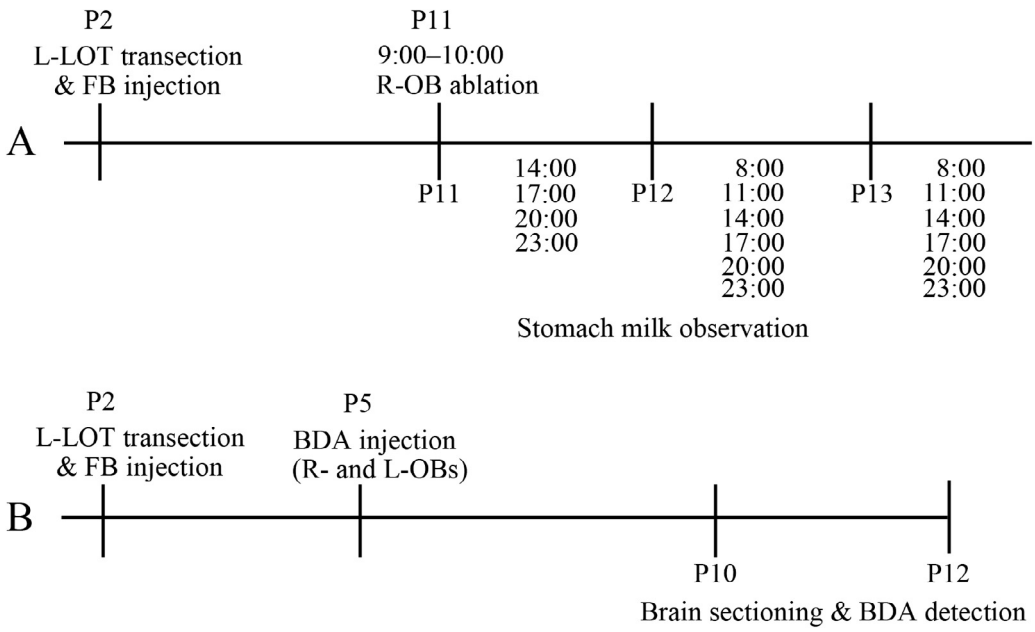

Fig. 1. Experimental designs of stomach milk observation (A) and BDA detection (B). OB: olfactory bulb.

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 h 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 exam-