

# **Regenerative Capacity of Bulbar Projection Neurons during Development: A Quantitative Neuronal Analysis with Functional Correlation**

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## **Abstract**

Critical periods and degrees of regeneration in injured olfactory bulbar projection neurons (mitral cells) were examined in adult rats whose lateral olfactory tracts (LOTs) were transected at different postnatal (P) days. After the LOTs were transected at P7, P10, and P14, a retrograde fluorescent tracer, Fluoro-Gold (FG), was injected into the posterior olfactory cortex (the olfactory tubercle and the piriform cortex), a target brain region of mitral cells, 5 weeks after the transection. FG (+) mitral cells were observed in P7 LOT-transected bulbs and some of P10 LOT-transected bulbs, but not in P14 LOT-transected bulbs. Neuron numbers of regenerated FG (+) mitral cells in P2 LOT-transected adult rats decreased to approximately 70% of the normal values (actually counted number:  $804 \pm 46$ ; stereologically estimated number:  $49,700 \pm 4,300$ ), and 100% of these rats were demonstrated to exhibit olfactory discriminative ability in our previous study. Meanwhile, the numbers in P7 LOT-transected adult rats further decreased to approximately 40% of the normal values, and 78% of these rats showed olfactory discriminative ability. We conclude that the critical periods of spontaneous regeneration of the LOT are between P0 and P10, and that the proportions of regenerated mitral cells decreased as rats became older.

**Key words:** mitral cells, lateral olfactory tract, regeneration, neuron numbers, olfaction

## **Introduction**

It is 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; Kikukawa et al. 1998; Ito et al. 1998; Sherrard and Bower 2001). The lateral olfactory tract (LOT), the main olfactory pathway that connects the olfactory bulb to the olfactory cortex (the olfactory tubercle and the piriform cortex) (Sekiguchi et al. 2012), was reported to undergo spontaneous regeneration 8 months after its transection in newborn rats (Munirathinam et al. 1997), whereas such regeneration does not occur in the adult stage. We recently reported that spontaneous regeneration of the LOT consistently occurred in newborn rats, and that the regenerated olfactory system was functional 4 weeks after its transection (Sakamoto et al. 2010), even when regenerated olfactory structures were incomplete in terms of myelination of the LOT and re-innervated cortical areas (Fukushima et al. 2013).

The present study was conducted to investigate two unresolved issues on the LOT regeneration: (1) the critical periods of spontaneous regeneration and (2) the proportions of regenerated bulbar projection neurons (mitral cells) with functional correlation. Two different fluorescent tracers were used for different purposes: one tracer was injected just after the LOT transection to exclude incomplete LOT-transected cases and

another tracer was injected 5 weeks and 12 weeks after the transection to visualize the tracer-labeled regenerated mitral cells. For quantification of regenerated mitral cells, stereological analyses were used for unbiased counting of mitral cells. Furthermore, for functional analysis of neonatally LOT-transected adult rats, they were subjected to the olfactory discriminative test after unilateral bulbectomy contralateral to the transection followed by retrograde neuronal tracing.

## **Materials and methods**

### **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 approved by our Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. Female newborn rat pups of the Wistar strain (Japan SLC Inc., Hamamatsu, Japan) were used for the experiments at different developmental stages. Postnatal day (P) 0 refers to the first 24 h after birth. A total of 53 pups and 15 adult (12-week-old female) rats were used in this study. Surgical manipulations on P2 ( $n = 7$ ), P7 ( $n = 32$ ), P10 ( $n = 7$ ), and P14 ( $n = 7$ ) pups were performed in a hypothermic condition using a freezer ( $-20^{\circ}\text{C}$ , 15-20 min). Rats

at more advanced ages were anesthetized by subcutaneous injection of a mixture of pentobarbital (50 mg/kg) and medetomidine (10 mg/kg). To reverse the effect of anesthesia, atipamezole hydrochloride (2 mg/kg) was injected intraperitoneally.

### **Spontaneous regeneration of the LOT during development**

#### *LOT transection combined with injection of a retrograde fluorescent tracer (FB)*

The LOT transection was performed in P7 ( $n = 7$ ), P10 ( $n = 7$ ) and P14 ( $n = 7$ ) pups unilaterally on the left side as described previously (Sakamoto et al. 2010; Sekiguchi et al. 2012; Fukushima et al. 2013). Briefly, LOT was transected at the posterior half of the olfactory stria by inserting a knife (Ophthalmic Scleral MVR Knife, 25 gauge; Alcon, Tokyo, Japan) from the ventrolateral aspect of the head. Immediately after the 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 the LOT transection (Sakamoto et al. 2010; Fukushima et al. 2013). FB (1% solution dissolved in distilled water, 0.1  $\mu$ l) was injected into the posterior part of the olfactory cortex situated far caudal to the site of the LOT transection. After surgery, the pups were housed with their dam. The dam and her pups were kept in a single cage (26  $\times$  42  $\times$  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.

*Injection of a different retrograde fluorescent tracer (FG) 5 weeks after LOT transection*

LOT-transected P7, P10 and P14 pups were allowed to survive for 5 weeks and received an injection of 0.4 µl of a different retrograde fluorescent tracer, Fluoro-Gold (FG, 4% solution dissolved in isotonic saline; Fluorochrome, Denver, CO, USA), into the posterior olfactory cortex on the left LOT-transected side. FG injections into the olfactory cortex were performed after the clear visualization of the ventrolateral aspect of the left brain. Two days after FG injections, the rats were deeply anesthetized with sodium pentobarbital (80-100 mg/kg, i.p.) and perfused through the heart with 150-200 ml of 4% paraformaldehyde 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 brain was serially cut into 30-µm thick sections in a sagittal plane at 180-µm intervals on a freezing microtome. The sections were serially mounted on coated slides, coverslipped with glycerol, and observed under a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan).

## **Quantification of FG (+) regenerated mitral cells during development**

*LOT transection at early (P2) and late (P7) neonatal stages followed by FG injection into the posterior olfactory cortex 12 weeks after LOT transection*

The LOT transection was performed in P2 ( $n = 7$ ) and P7 ( $n = 7$ ) pups unilaterally on the left side combined with FB injection into the posterior olfactory cortex as described previously. These LOT-transected pups were allowed to survive for 12 weeks, and received stereotaxic injections of 0.5  $\mu$ l of FG into the posterior olfactory tubercle (0.5 mm anterior from the bregma, 2.5 mm lateral from the midline, and 8.0 mm deep from the dura mater; in reference to “the rat brain in stereotaxic coordinates” by G. Paxinos and C. Watson) and 0.5  $\mu$ l of FG into the posterior piriform cortex (0.5 mm anterior from the bregma, 4.5 mm lateral from the midline, and 7.0 mm deep from the dura mater) on the left LOT-transected side. Normal 12-week-old female rats ( $n = 5$ ) received similar stereotaxic injections of FG into the left posterior olfactory cortex. Two days after FG injections, the rats were deeply anesthetized, and perfused through the heart with 250-300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, postfixed overnight in the same fixative, and soaked in 30% sucrose for 2 days.

### *Tissue preparation and immunohistochemistry for FG*

The brain was cut into three pieces with the left and right olfactory bulbs, and the remaining brain. The left olfactory bulb was serially cut into 50- $\mu$ m thick sections in a coronal plane serially at 400- $\mu$ m intervals (every eighth sections) on a freezing microtome. After cutting the lateral half of the right brain, the left brain was serially cut into 50- $\mu$ m thick sections in a coronal plane at 600- $\mu$ m intervals (every twelfth sections) on a freezing microtome. 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. They were incubated in biotinylated anti-guinea pig immunoglobulins (1:500; Dako, Glostrup, Denmark) for 1 h, and then in streptavidin-peroxidase (1:400; Dako) for 1 h. The sections were visualized with Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific Inc., Pierce Biotechnology, Rockford, USA). The sections were serially mounted on coated slides and coverslipped.

### *Neuronal counting of FG (+) mitral cells*

FG (+) mitral cells in the bulbar serial sections of the normal, P2 LOT-transected, and



P7 LOT-transected adult rats were counted. To count the number of FG (+) mitral cells, a three-dimensional dissector was placed in each section using the systematic random sampling method, which is commonly used in stereology. In detail,  $100 \times 100 \mu\text{m}^2$  grids were systematically and randomly placed over the mitral cell layer with the commercially available stereological software (Stereoinvestigator; MBF Bioscience, VT, USA). At regularly predetermined positions of the grid, cells were counted within three-dimensional optical dissectors with a  $20\times/0.50$  UPlanFI objective. Parameters of stereology were summarized in Table 1. Within each dissector ( $50 \times 50 \times 15 \mu\text{m}$ ), FG (+) mitral cells with diameters larger than  $15 \mu\text{m}$  were counted (Figure 1) (McCollum et al. 1997; Koyano et al. 2005). Neuronal counting was performed three times on each bulb, and mean values of both actually counted and stereologically estimated FG (+) mitral cells were regarded as total numbers of FG (+) mitral cells in each rat. Quantification of FG (+) mitral cells in the normal, P2 LOT-transected, and P7 LOT-transected groups was performed, and the data were expressed as mean  $\pm$  SD. Statistical significance of the means was evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni test as a post hoc analysis. *P* values less than 0.05 were considered significant.

### **Functional analysis followed by retrograde neuronal tracing**

Since pups whose LOTs were transected at the early neonatal stage (P2) have been shown to exhibit olfactory discriminative ability at the later age of 4 weeks, pups ( $n = 18$ ) whose LOTs were transected at the late neonatal stage (P7) underwent the olfactory discriminative test 12 weeks after LOT transection. Before the test, to investigate the functioning of the left olfactory system of rats that underwent neonatal LOT transection on the left side, the right olfactory bulb was ablated by aspiration with a 21-gauge needle. Besides neonatally LOT-transected adult rats, 12-week-old female rats were subjected to unilateral ( $n = 5$ ) or bilateral ( $n = 5$ ) bulbectomy to confirm the reliability of the test. After deprivation of water for 2-3 days, the rats were subjected to an odor aversion behavior test to examine their olfactory ability to discriminate between 0.01% cycloheximide solution and water by sniffing, as described in our previous reports (Moriizumi et al. 1994; Fukushima et al. 2002; Sakamoto et al. 2010; Sekiguchi et al. 2012). Because the taste of cycloheximide solution is considerably unpleasant, rats memorized the smell of the solution upon drinking, and thereafter learned to avoid it by olfaction. When rats drank the water, the response was interpreted as a correct response. When rats drank the cycloheximide solution, the response was interpreted as an incorrect 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 experimental groups. Data are expressed as mean  $\pm$  SD. Statistical significance of the means was evaluated by ANOVA followed by the Dunnett test as a post hoc analysis. *P* values less than 0.05 were considered significant. After the olfactory discrimination test, a part ( $n = 4$ ) of neonatally LOT-transected adult (12-week-old) rats received stereotaxic injections of 0.5  $\mu$ l of FG into the posterior olfactory tubercle and 0.5  $\mu$ l of FG into the posterior piriform cortex on the left LOT-transected side. Two days after FG injections, the rats were deeply anesthetized and perfused through the heart with 250-300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The frozen sections from the left olfactory bulb and the left brain were processed for immunohistochemical analysis for FG, and quantification of FG (+) mitral cells was performed in the aforementioned manner.

## **Results**

### **Spontaneous regeneration of the LOT during development**

Figure 2A shows representative injection sites of two different fluorescent tracers that were injected into the posterior olfactory cortex at different developmental stages. FB was injected on P7 just after transection of the LOT, whereas FG was injected 5 weeks

later. Olfactory bulbs from P7 LOT-transected 6-week-old rats ( $n = 5$ ) contained a substantial number of FG (+) cells in the mitral cell layer (Figures 2B and 2C). Olfactory bulbs from P10 LOT-transected 6-week-old rats ( $n = 6$ ) were divided into two groups, those with no FG (+) mitral cells ( $n = 3$ ) and those with a very small number of FG (+) mitral cells ( $n = 3$ ). Neuronal labeling by FG was not present in the bulbar mitral cell layer from P14 LOT-transected 7-week-old rats ( $n = 6$ ) (Figure 2D). Incomplete LOT-transected cases with numerous FB (+) and FG (+) cells in the mitral cell layer were excluded from the experiments ( $n = 4$ ).

### **Quantification of FG (+) regenerated mitral cells during development**

Figures 3A and 3B show macroscopic views of brains from P2 and P7 LOT-transected adult rats. The white myelinated bands of the LOT were lost from the retrobulbar region, the presumed LOT transection sites performed at the neonatal stages. Representative FG injection sites at the adult stage are shown in Figure 4. The tracer diffused to the caudal halves of the olfactory tubercle and the piriform cortex in all cases. However, in some cases with relatively larger injections of the tracer, the caudal two-third of the olfactory tubercle and/or the caudal two-third of the piriform cortex were involved in FG diffusion. The tracer did not involve the rostral one-thirds of the

olfactory tubercle and the piriform cortex in all cases. Figures 5A-5I show rostrocaudal distributions of FG (+) mitral cells from a normal 12-week-old rat. Figure 6 shows microscopic profiles of FG (+) mitral cells from normal (6A and 6D), P2 LOT-transected (6B and 6E), and P7 LOT-transected (6C and 6F) adult rats. FG (+) mitral cells of the normal and P2 LOT-transected groups were widely distributed within all bulbar sections without specific localizations in the medial, lateral, ventral or dorsal bulb. FG (+) mitral cells of the P7 LOT-transected group showed marked reduction in number, but they were observed within all bulbar sections without specific intrabulbar localizations. Table 1 shows stereological analyses on total numbers of FG (+) mitral cells both counted (mean from three separate actually counted values) and estimated (mean from three separate stereologically estimated values) in individual cases from all experimental groups. Total numbers of FG (+) mitral cells from the normal group were  $804 \pm 46$  (counted) and  $49,700 \pm 4,300$  (estimated), respectively. Total numbers of the cells from the P2 LOT-transected group decreased to  $561 \pm 53$  (counted) and  $35,100 \pm 4,500$  (estimated), respectively, while total numbers of the cells from the P7 LOT-transected group further decreased to  $327 \pm 76$  (counted) and  $19,700 \pm 5,000$  (estimated), respectively. Significant differences in total neuron numbers were found among these three groups ( $P < 0.01$ ) (Figures 7 and 8). Incomplete

LOT-transected cases with FB (+) and FG (+) cells in the mitral cell layer were excluded from the experiments ( $n = 4$ ).

### **Functional analysis followed by retrograde neuronal tracing**

Neonatally (P7) LOT-transected adult rats ( $n = 18$ ) were examined for olfactory function on the left LOT-transected side after removal of the right olfactory bulb. In terms of correct responses, these rats were divided into two groups. The first group ( $n = 14$ ) gave one to four incorrect responses in the first set of 10 trials, but thereafter they showed aversive behavior to the cycloheximide solution. Their correct responses greatly increased in the second and third sets of 10 trials. The percentage of correct responses in this group was  $90\% \pm 3\%$  in all trials and  $99\% \pm 3\%$  in the last 10 trials, and no significant differences in the percentage ( $91\% \pm 1\%$  in all trials and  $100\% \pm 0\%$  in the last 10 trials) of correct responses in the unilateral bulbectomy group ( $n = 5$ ) were found (Figure 9). The second group ( $n = 4$ ) did not show aversive behavior to the cycloheximide solution during the test. The percentage of correct responses in this group was  $50\% \pm 2\%$  in all trials and  $50\% \pm 2\%$  in the last 10 trials, and no significant differences in the percentage ( $50\% \pm 2\%$  in all trials and  $49\% \pm 3\%$  in the last 10 trials) of correct responses in the bilateral bulbectomy group ( $n = 5$ ) were found (Figure 9).

Two rats from the first group and two rats from the second group that received stereotaxic injections of FG into the posterior olfactory cortex were examined for quantification of FG (+) mitral cells. Stereological analyses on total numbers of FG (+) mitral cells in individual cases are shown in Table 1. Total numbers of the cells from the rats with olfactory discriminative ability (olfaction (+)) were 328 and 294 (counted), and 23,400 and 21,300 (estimated), respectively (Figure 10A), while total numbers of the cells from the rats without olfactory discriminative ability (olfaction (-)) were 173 and 137 (counted), and 13,000 and 9,600 (estimated), respectively (Figure 10B). These four rats were confirmed as having no FB (+) cells in the mitral cell layer.

## **Discussion**

### **Critical periods of spontaneous regeneration of the LOT**

In regeneration experiments of the brain fiber tract, it is very important that transection of the fiber tract should be completely performed. Therefore, a retrograde fluorescent tracer (FB) was simultaneously injected at the time of the LOT transection to exclude incomplete LOT-transected cases where FB was transported to uninjured mitral cells in the olfactory bulb. In complete LOT-transected cases with no FB (+) mitral cells,

retrograde FG labeling was consistently observed in P7, but not P14 LOT-transected bulbs. Sparse FG labeling occurred in half of P10 LOT-transected bulbs, whereas no FG labeling occurred in the other half. Therefore, we conclude that the critical periods of spontaneous regeneration of the LOT are between P0 and P10. Similar critical periods (postnatal one week) of spontaneous regeneration were reported in the rat olivocerebellar projection (Sherrard and Bower 2001).

### **Proportion of regenerated bulbar projection neurons**

Although FG (+) mitral cells could be observed under a fluorescent microscope, we converted FG into a DAB-reactive product using immunohistochemical procedures with an anti-FG antibody; thus, FG (+) mitral cells could be repeatedly observed under a bright field microscope without the problem of fluorescence fading. Random sampling by the stereological software enabled us to perform unbiased neuronal counting of FG (+) mitral cells. Total numbers of regenerated FG (+) mitral cells were different depending on the time of the LOT transection at the early (P2) and late (P7) neonatal stages. The mean values of total numbers of FG (+) mitral cells from P2 and P7 LOT-transected adult bulbs decreased to 70% (counted) - 71% (estimated) and 40% (estimated) - 42% (counted) of the normal corresponding values (counted: 804;



estimated: 49,700), respectively. The present study provided quantitative data on gradual decreases in the proportion of regenerated bulbar projection neurons during postnatal development. To our knowledge, no data on the proportion of regenerated projection neurons in the central nervous system are available. The results of this study indicate that the capability of bulbar projection neurons to extend their injured axons across the transected site far caudally to their original cortical target regions is higher in mitral cells from P2 LOT-transected bulbs than in those from P7 LOT-transected bulbs. It could be speculated that phenomenon of the different degrees of spontaneous regeneration of the LOT may be owing to postnatal increases in inhibitory molecules that suppress axon elongation (Sijbesma and Leonard 1986; Rhodes and Fawcett 2004; Laabs et al. 2005; Busch and Silver 2007; Lee and Zheng 2012).

### **Regenerated neuron numbers in relation to olfactory function**

Previous reports (Devor 1975; Small and Leonard 1983; Ito et al. 1998) dealing with functional recovery by regenerated neurons are very few, particularly with regard to the central nervous system. We have reported in our previous paper (Sakamoto et al. 2010) that the regenerated olfactory system was functional 4 weeks after the LOT

transection at P2, and the present study has further revealed that the olfactory discriminative ability was achieved by regenerated bulbar projection neurons that constitute approximately 70% of the normal value. A total of 100% (20/20) of adult rats whose LOTs were transected at P2 showed such olfactory discriminative ability (Sakamoto et al. 2010), while 78% (14/18) of adult rats whose LOTs were transected at P7 showed similar ability, where the proportion of regenerated bulbar projection neurons decreased to approximately 40% of the normal value. From a functional point of view, the proportion of regenerated bulbar projection neurons of the two P7 LOT-transected rats with olfactory discriminative ability was 41% (counted) - 47% (estimated) and 37% (counted) - 43% (estimated) of the normal corresponding values, while that of the neurons of the two P7 LOT-transected rats without olfactory discriminative ability was 22% (counted) - 26% (estimated) and 17% (counted) - 19% (estimated) of the normal corresponding values. We reported that rats with undamaged healthy mitral cells of less than 22% of the unilateral normal value lacked olfactory discriminative ability in acute LOT-lesioning experiments (Fukushima et al. 2002). Interestingly, Lu and Slotnick (1998) reported that rats with bulbar savings scores of less than 21% had deficits in olfactory detection or discrimination tasks. We alleged the importance of the functionally essential neuronal population, which implies

a critical value for the preservation of adequate neural functioning. Neuronal population required for olfactory functional preservation was reported to approximately 50% of neonatal olfactory receptor neurons (Kawagishi et al. 2009).

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## Figure legends

**Figure 1** An example of a three-dimensional optic dissector that is composed of square and counting depth (asterisk). The mitral cell layer of the olfactory bulb is encircled by long parallel lines. A cross on the screen is 15  $\mu\text{m}$  in size and used for marking cells (dots) with diameters larger than 15  $\mu\text{m}$ .

**Figure 2** (A-C) A P7 LOT-transected 6-week-old rat. (A) Injection sites of FB and FG into the olfactory cortex (OC). FB was injected at P7 immediately after the LOT transection, while FG was injected 5 weeks later. (B, C) Note the absence of FB (+) cells and the presence of FG (+) cells in the bulbar mitral cell layer. (D) A P14 LOT-transected 7-week-old rat. FB was injected at P14 immediately after the LOT transection, while FG was injected 5 weeks later. No FG (+) cells were found in the olfactory bulb.

**Figure 3** Ventral views of brains from a P2 LOT-transected 12-week-old rat (A) and a P7 LOT-transected 13-week-old rat (B). Note the disappearance of the left LOTs on

the transected side. Arrows indicate the presumed sites of the LOT transection at the neonatal stages. Asterisks point to intact LOTs on the right side.

**Figure 4** Injection sites of FG into the olfactory cortex (OC) in a normal 12-week-old rat. Medial and lateral arrows point to the olfactory tubercle and the piriform cortex, respectively.

**Figure 5 (A-I)** Sequential coronal views of an olfactory bulb from a normal 12-week-old rat that received FG injections into the olfactory cortex. Note the clearly visible mitral cell layers consisting of numerous FG (+) cells. An asterisk indicates the accessory olfactory bulb. Arrowheads point to the mitral cell layer in the caudal olfactory bulb. Bar: 500  $\mu$ m.

**Figure 6** Olfactory bulbs from normal (**A, D**), P2 (**B, E**) and P7 (**C, F**) LOT-transected adult rats that received FG injections into the olfactory cortex. Olfactory bulbs in **A-C** are taken from the bulbar central portions just rostral to the accessory olfactory bulb. Photomicrographs in **D-F** show FG (+) mitral cells at higher magnification. Note the gradual decreases in FG (+) mitral cells from normal to P2

LOT-transected bulbs, and from P2 to P7 LOT-transected bulbs. Bars: (A-C) 500  $\mu\text{m}$ ;  
(D-F) 50  $\mu\text{m}$ .

**Figure 7** Total neuron numbers of FG (+) mitral cells counted in normal, P2  
LOT-transected and P7 LOT-transected groups. \*\*  $P < 0.01$

**Figure 8** Total neuron numbers of FG (+) mitral cells estimated in normal, P2  
LOT-transected and P7 LOT-transected groups. \*\*  $P < 0.01$

**Figure 9** Correct responses of the four groups of the experimental animals as  
expressed by percentages in the last 10 trials. (A) Rats with unilateral bulbectomy  
(100%). (B) P7 LOT-transected rats with olfactory discriminative ability (99%). (C)  
P7 LOT-transected rats without olfactory discriminative ability (50%). (D) Rats with  
bilateral bulbectomy (49%). \*\*  $P < 0.01$  vs. unilateral bulbectomy.

**Figure 10** FG (+) mitral cells from P7 LOT-transected adult rats with olfactory  
discriminative ability (A) and without olfactory discriminative ability (B). Their total  
neuron numbers are 328 (counted) and 23,400 (estimated), and 137 (counted) and 9,600

(estimated), respectively. Bar: 50  $\mu\text{m}$ .

**Table 1** Stereological analyses of FG (+) mitral cells

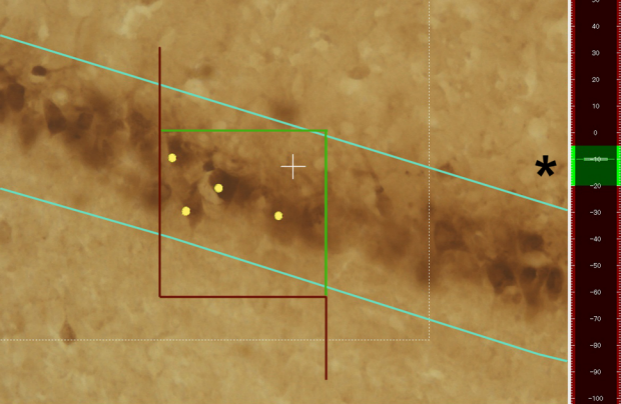
Experimental groups	Normal					P2 LOT-transected				
Animal No.	1	2	3	4	5	1	2	3	4	5
Number of sections	7	10	9	10	9	8	9	10	10	10
Number of sampling sites	192	242	254	259	254	195	230	211	234	180
Mean measured section thickness ( $\mu\text{m}$ )	30.5	30.0	26.9	29.4	27.9	29.8	28.4	30.0	30.8	27.8
Total number of FG (+) mitral cells counted	764	882	791	807	777	600	559	565	605	474
Total number of FG (+) mitral cells estimated	49,800	56,200	45,400	50,800	46,300	38,200	33,800	35,500	39,700	28,100
CE Gundersen ( $m = 1$ )	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05
Experimental groups	P7 LOT-transected					Olfaction (+)		Olfaction (-)		
Animal No.	1	2	3	4	5	6	7	8	9	
Number of sections	9	7	9	9	8	9	7	7	8	
Number of sampling sites	192	164	192	165	190	202	142	166	189	
Mean measured section thickness ( $\mu\text{m}$ )	27.7	27.5	29.7	27.5	27.0	33.7	33.1	37.6	33.1	
Total number of FG (+) mitral cells counted	408	327	220	274	443	328	294	173	137	
Total number of FG (+) mitral cells estimated	23,900	19,200	13,800	16,100	25,400	23,400	21,300	13,000	9,600	
CE Gundersen ( $m = 1$ )	0.05	0.06	0.07	0.06	0.05	0.06	0.06	0.08	0.09	

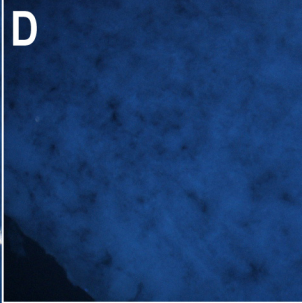
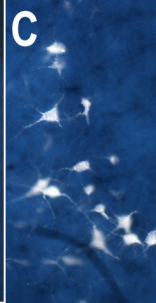
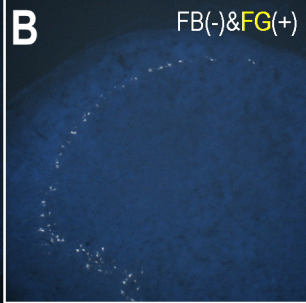
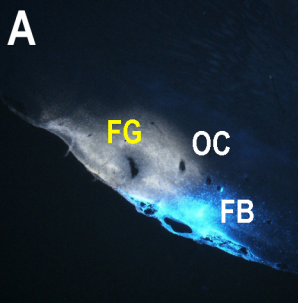
## Common settings

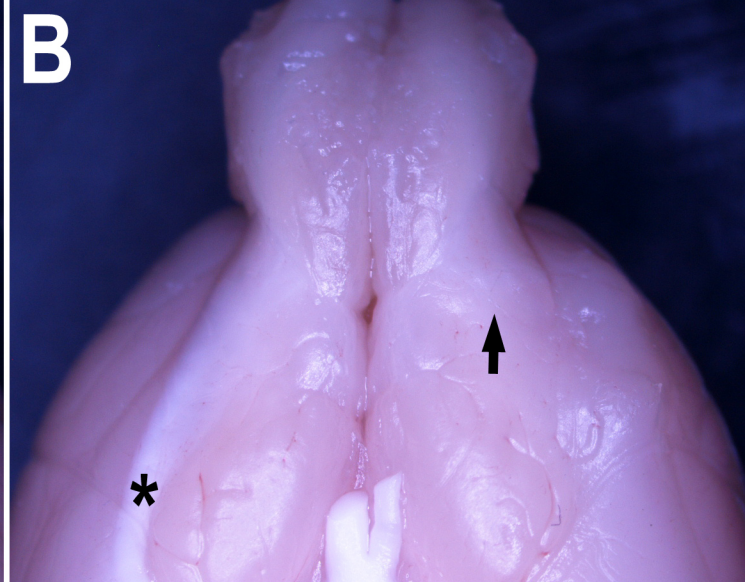
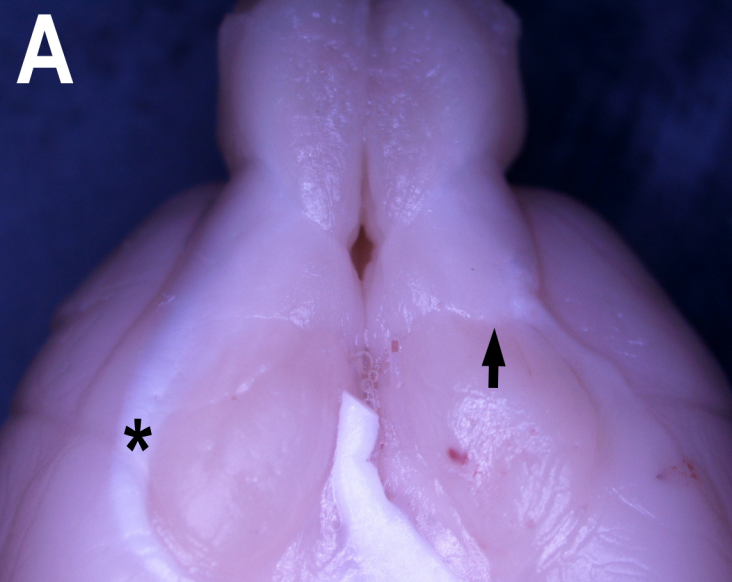
Section evaluation interval: 8; Sampling grid area: 100  $\mu\text{m}$  x 100  $\mu\text{m}$ ; Counting frame area: 50  $\mu\text{m}$  x 50  $\mu\text{m}$ ;

Dissector height: 15  $\mu\text{m}$ ; Safe guard zone height: 5  $\mu\text{m}$ .

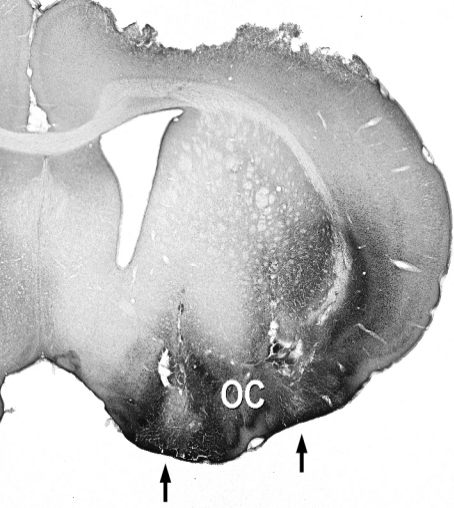
FG (+) mitral cells with diameters larger than 15  $\mu\text{m}$  were counted.

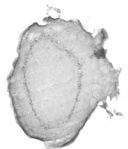
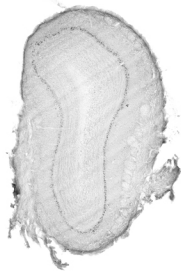
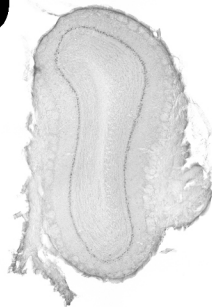
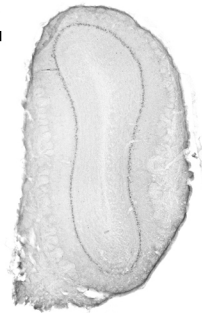
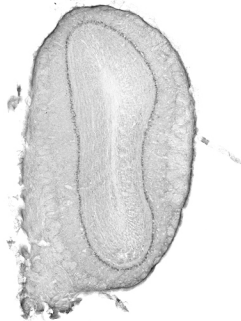
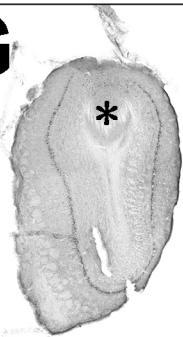
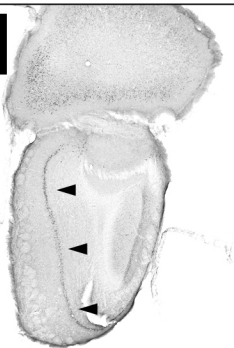
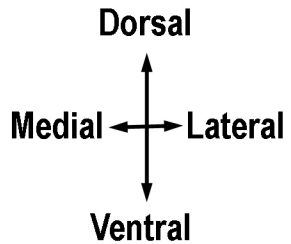
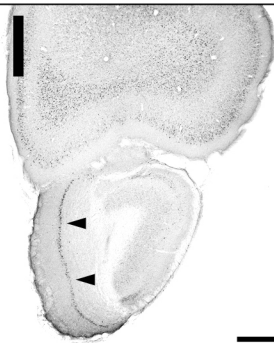


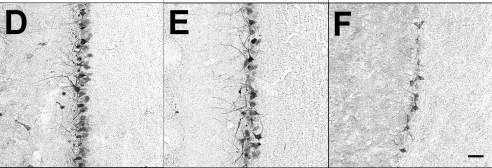
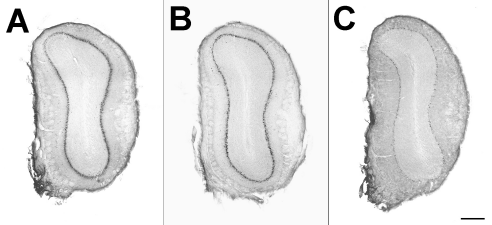


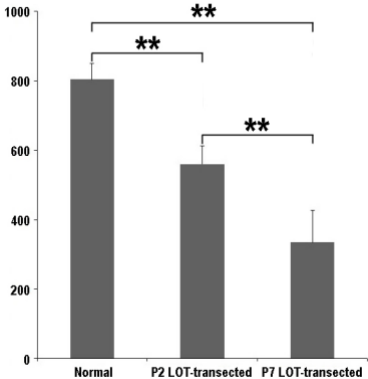


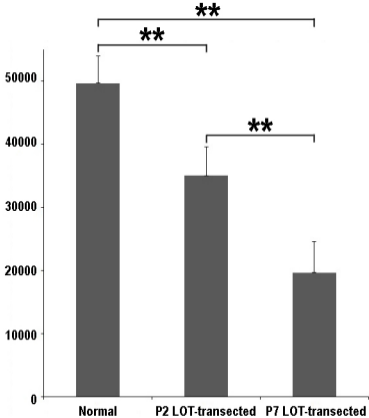


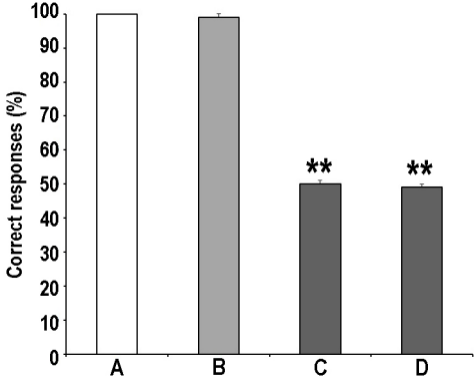


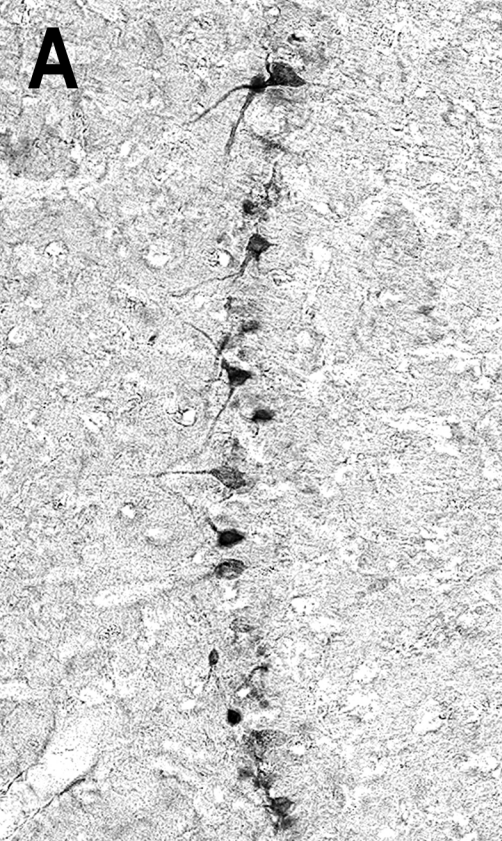
**A****B****C****D****E****F****G****H****I**









**A****B**