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The Minimum Number of Neurons in the Central Olfactory Pathway in Relation to its Function: a Retrograde Fiber Tracing Study

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Abstract

The present study was aimed at determining the functionally essential size of the neuronal population in the central olfactory nervous system. Using conditioned rats who had learnt to avoid repellent (cycloheximide) solution by olfaction, varying degrees of injuries were made to the lateral olfactory tract, a major central olfactory pathway connecting the olfactory bulb to the olfactory cortex. After examining their olfactory ability to discriminate cycloheximide solution from water, intact bulbar projection neurons (mitral cells) with fiber connections to the olfactory cortex were quantified using a retrograde fiber tracing technique. The numbers of retrogradely labeled mitral cells from the rats with normal olfaction ranged between 20 and 92% of the control value, while those numbers from the anosmic rats ranged between 0 and 22%. We conclude that the functionally essential neuronal population is approximately one-fifth of the total in the central olfactory pathway, a presumed threshold value in terms of the ability to avoid cycloheximide solution by olfactory discrimination.

Introduction

Sensation is perceived by neuronal networks from sensory organs to the brain. In the brain, many central sensory pathways transmit peripheral sensory information to specific cortical areas, depending on the different senses. However, the minimal number of sensory neurons and fibers (central sensory pathways) needed for a specific sensation has yet to be determined.

The olfactory nervous system is a hierarchically ordered neuronal pathway that consists of three main stations: the olfactory epithelium, olfactory bulb and olfactory cortex. Olfactory receptor neurons in the olfactory epithelium, transforming chemical stimuli to electric signals, send their axons (olfactory nerves) to the olfactory bulb and make synapses with bulbar projection neurons (mitral cells) (Lancet, 1988; Buck and Axel, 1991). The mitral cells, developmentally similar to cortical pyramidal neurons, are the biggest neurons in the bulb, and project to the olfactory cortex via the lateral olfactory tract (LOT) (Scott, 1986; Greer, 1991; Kratskin, 1995; Moriizumi *et al*., 1995). Thus the LOT is a major central olfactory pathway connecting the olfactory bulb to the olfactory cortex, and is made up of myelinated fiber bundles of mitral cells.

We have already established a simple and accurate method to examine olfactory function in rodents by odor aversion behavior (Kimura *et al*., 1991; Moriizumi *et al*., 1994). This is based on the ability to discriminate repellent (cycloheximide) solution from water by olfaction. Since its taste is so disgusting, rodents remember the smell of the solution when they drink it, and thereafter learn to avoid it by olfaction. Thus an olfactory conditioned reaction is established that enables us subsequently to examine olfactory function.

The present study was undertaken to determine neuroanatomically the minimum number of mitral cells required for the ability to avoid cycloheximide solution by olfaction. For this purpose, a retrograde neuronal tracer was employed to quantify the numbers of intact mitral cells with fiber connections to the olfactory cortex among LOT-lesioned rats that were functionally divided into two groups: one with normal olfaction and one with loss of olfaction in terms of cycloheximide solution.

Materials and methods

The experiments were carried out on adult male Wistar rats (11–13 weeks old, 250–300 g). All surgical manipulations were done under general anesthesia with sodium pentobarbital (50 mg/kg, i.p.). First, unilateral olfactory bulbs were completely ablated by aspiration with a 21G needle. The unilaterally bulbectomized rats (n = 31) were trained to avoid cycloheximide solution. The detailed experimental procedures of the olfactory discrimination test were similar to those in our previous paper (Moriizumi *et al*., 1994) but with the slight modification mentioned below. When the rat sniffed the cycloheximide solution and drank it, the

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response was interpreted as a wrong response. When the rat sniffed the cycloheximide solution, avoided it and drank water, the response was regarded as a correct response. The number of correct responses was divided by the number of total responses, and the percentage of correct responses was calculated. All rats were successfully conditioned to avoid cycloheximide solution, and gave 100% correct responses.

Transection of the LOT ipsilateral to the remaining olfactory bulb was performed in the conditioned rats (n = 25). The LOT transection was produced by aspiration with a 27G needle. Retrospective examination showed that the LOT lesions were localized between 2–4 mm from the posterior end of the bulb. In the control rats (n = 6), the LOT was exposed and no lesions were made on it. After deprivation of water for 2–3 days, the rats were submitted to odor aversion behavior to examine their olfactory ability to discriminate cycloheximide solution from water. Then correct responses were estimated for each rat from the control and LOT-lesioned groups.

After the discrimination test, the rats received injections of Fluoro-Gold (FG), a fluorescent retrograde neuronal tracer, into the olfactory cortex (piriform cortex) on the side of the remaining olfactory bulb. A relatively large volume (1.5 μl) of 2% FG (Fluorochrome, Denver, CO) dissolved in distilled water was injected to cover the whole olfactory cortex. The animals were allowed to survive for 4 days. They were then deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the heart with 350–400 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 olfactory bulb and the olfactory cortex were cut serially at 30 μm thickness on a freezing microtome (olfactory bulb: horizontal plane; olfactory cortex: coronal plane). Parts of the sections from the control rats were counterstained with propidium iodide (PI) (20 μg/ml) (Sigma, St Louis, MO), because PI visualized the total mitral cell populations. The sections were mounted and examined under a fluorescence microscope. In the olfactory cortex where FG was injected, care was taken to examine whether the tracer involved the whole olfactory cortex, which was easily identified by its characteristic S-shaped configuration of the pyramidal cell layer. In cases where the tracer involved only part of the olfactory cortex and did not cover the whole olfactory cortex, these animals were excluded from the experiments.

In the olfactory bulb, the numbers of FG-positive mitral cells were counted in the three bulbar sections. These sections were selected from the center of the upper one-third, middle one-third and lower one-third portions of the bulb. After measuring the length of the mitral cell layer in each section, the average number of FG-positive mitral cells per unit length (mm) of the mitral cell layer was calculated in each animal. Thus the numbers of mitral cells with fiber connections to the olfactory cortex were determined in the control rats, the LOT-lesioned rats with normal olfaction and those rats with anosmia. The experiments were carried out in accordance with institutional guidelines for the care and use of animals established by the Animal Care and Use Committee at Shinshu University, and every effort was made to minimize animal suffering and pain.

Results

Since unilateral bulbectomy does not affect odor aversion learning, as shown previously (Moriizumi *et al.*, 1994), rats (n = 31) were subjected to the unilateral removal of an olfactory bulb to assess relationships between lesions of the LOT, olfactory function and numbers of lesion-sparing mitral cells by limiting the examination of the olfactory system to one side. The unilaterally bulbectomized rats were conditioned to avoid cycloheximide solution and gave 100% correct responses, as mentioned previously. The control rats (n = 6) remained intact after the LOT was exposed (Figure 1A). The other rats (n = 25) were then subjected to transection of the LOT ipsilateral to the remaining olfactory bulb, and varying degrees of lesions were made on the LOT (Figure 1B).

After deprivation of water, the rats were subjected to the discrimination test. The control rats gave 90–100% (97 ± 5%) correct responses. The LOT-lesioned rats were sharply divided into two groups. One group (n = 7) showed 90–100% (94 ± 5%) correct responses, indicating normal olfaction. The other group (n = 18) showed 0–20% (3 ± 7%) correct responses, indicating loss of olfaction. To confirm soundness of the discrimination test as a method of examining olfactory function, we removed the remaining olfactory bulb in the unilaterally bulbectomized, conditioned rats (n = 3).

Figure 1 The olfactory bulb, lateral olfactory tract and olfactory cortex. **(A, B)** Ventrobasal view of the forebrain from control (A) and lesioned (B) rats. The lateral olfactory tract (LOT) can be seen as a white continuous band (arrowheads), and can be traced from the olfactory bulb (OB) towards the olfactory cortex, just lateral to the optic chiasma (OC) and optic tract. A well-localized lesion is made on the LOT (an arrowhead). **(C)** Cytoarchitecture of the olfactory cortex (piriform cortex) in the basal aspect of the temporal lobe. Note the S-shaped regular array of pyramidal neurons, a characteristic feature of the olfactory cortex (arrowheads). Cresyl violet stain. **(D)** A representative injection site of the fluorescent tracer (Fluoro-Gold, FG) into the ventral part of the temporal cortex including the olfactory cortex. **(E)** Cytoarchitecture of the olfactory bulb with six layers. Mitral cells are located in the center of the bulb and form a distinct cell layer (arrowheads). Cresyl violet stain. **(F)** A mitral cell layer. Notice numerous mitral cells that are retrogradely labeled with FG injected into the olfactory cortex. Scale bars: (A, B) = 5 mm; (C, D) = 1 mm; (E) = 1 mm; (F) = 100 μm.

Figure 2 Note the overlapping of FG-positive cells with propidium iodide (PI)-positive mitral cells in a control rat. FG injections into the olfactory cortex. PI stain. Scale bar: 50 μm.

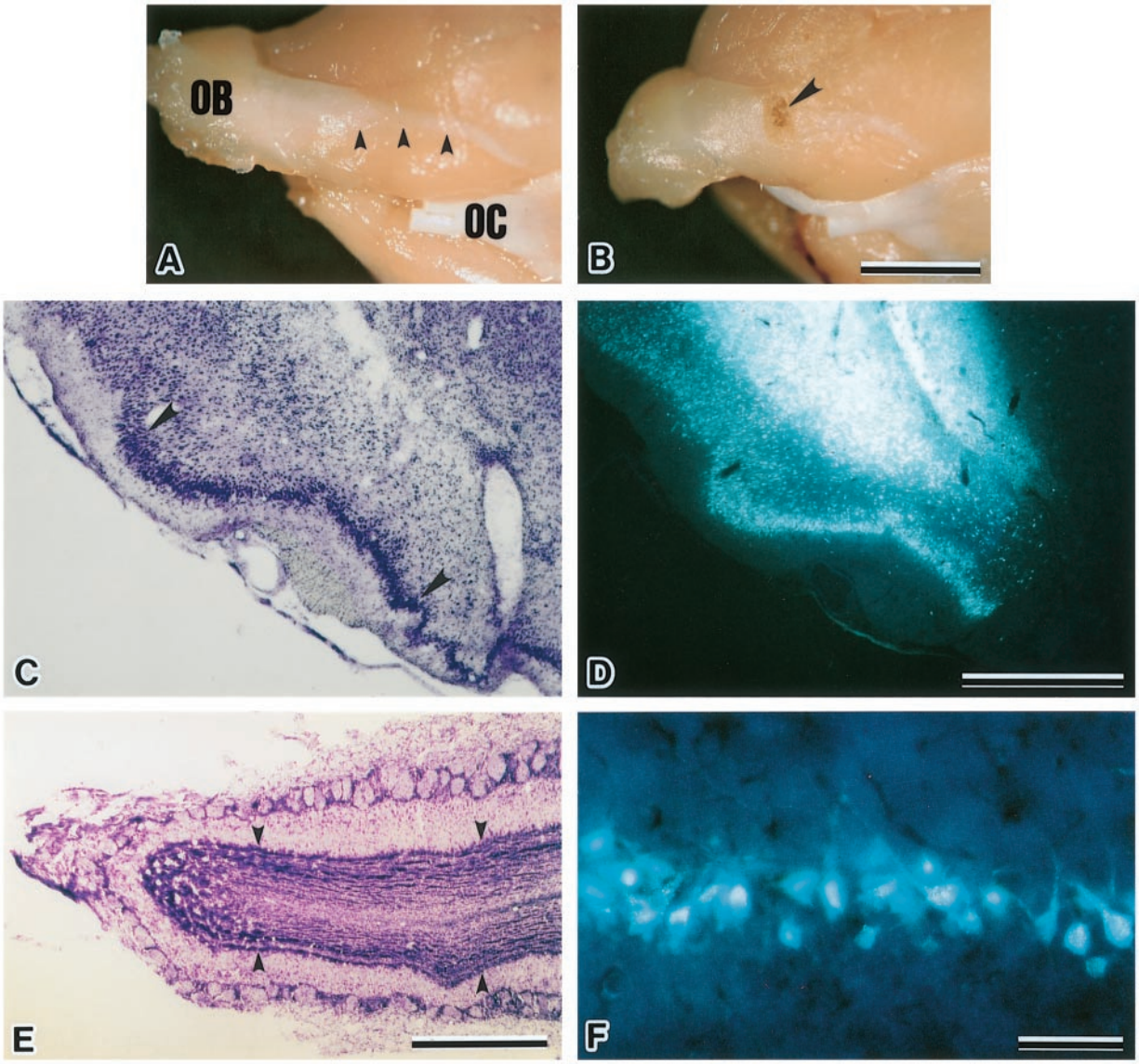

Figure 1

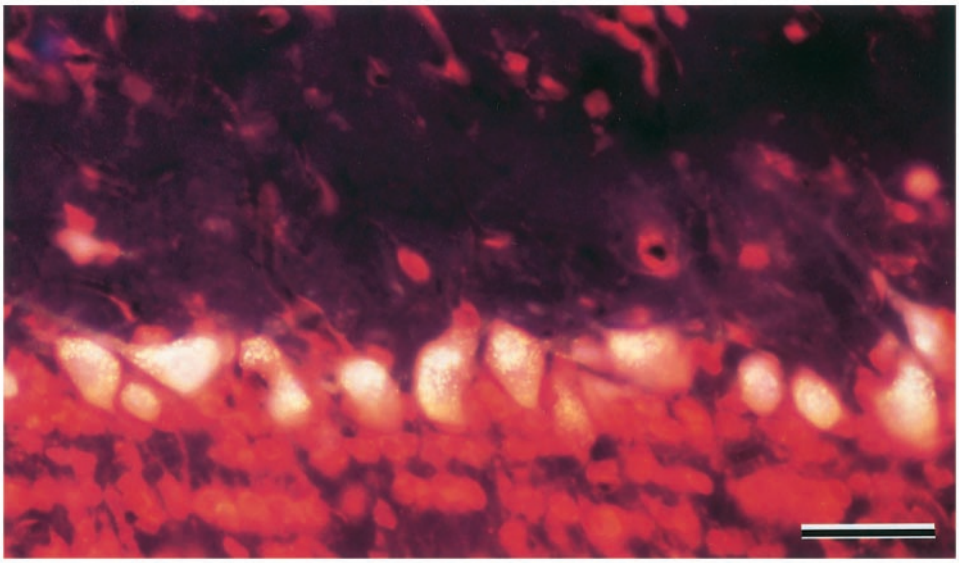

Figure 2

These rats with bilateral bulbectomy gave 0% correct responses. Following the experimental procedures mentioned in detail in our previous paper (Moriizumi *et al.*, 1994), we used 0.01% cycloheximide solution to examine the olfactory function of the experimental animals throughout this study. The anosmic rats with the LOT lesions (n = 8) were also examined with the higher concentrations (0.05 and 0.1%) of cycloheximide solution and water, and were unable to discriminate between these two solutions.

After the discrimination test, the rats received injections of FG into the olfactory cortex (piriform cortex) on the side of the remaining olfactory bulb to obtain quantitative data about mitral cell populations with fiber connections to the olfactory cortex (Figure 1C,D). FG injections into the olfactory cortex resulted in total labelings of mitral cells with FG that was retrogradely transported from the olfactory cortex (Figure 1E,F). PI staining also confirmed that the FG-positive cells completely matched the PI-positive mitral cells (Figure 2). The tufted cells in the external plexiform layer were not labeled with FG, except for a few deep tufted cells in the vicinity of the mitral cell layer (Figures 1F, 2 and 3). In the control rats, numerous mitral cells were labeled with FG, forming a well-defined mitral cell layer (Figure 3A,B). In contrast, FG-positive mitral cells were reduced in number in the LOT-lesioned rats. No preferential distribution of those FG-positive cells was found in the bulb. Compared with the LOT-lesioned rats with normal olfaction (Figure 3C,D), numbers of FG-positive mitral cells were much lower in the LOT-lesioned rats with anosmia (Figure 3E,F). The average numbers of FG-positive mitral

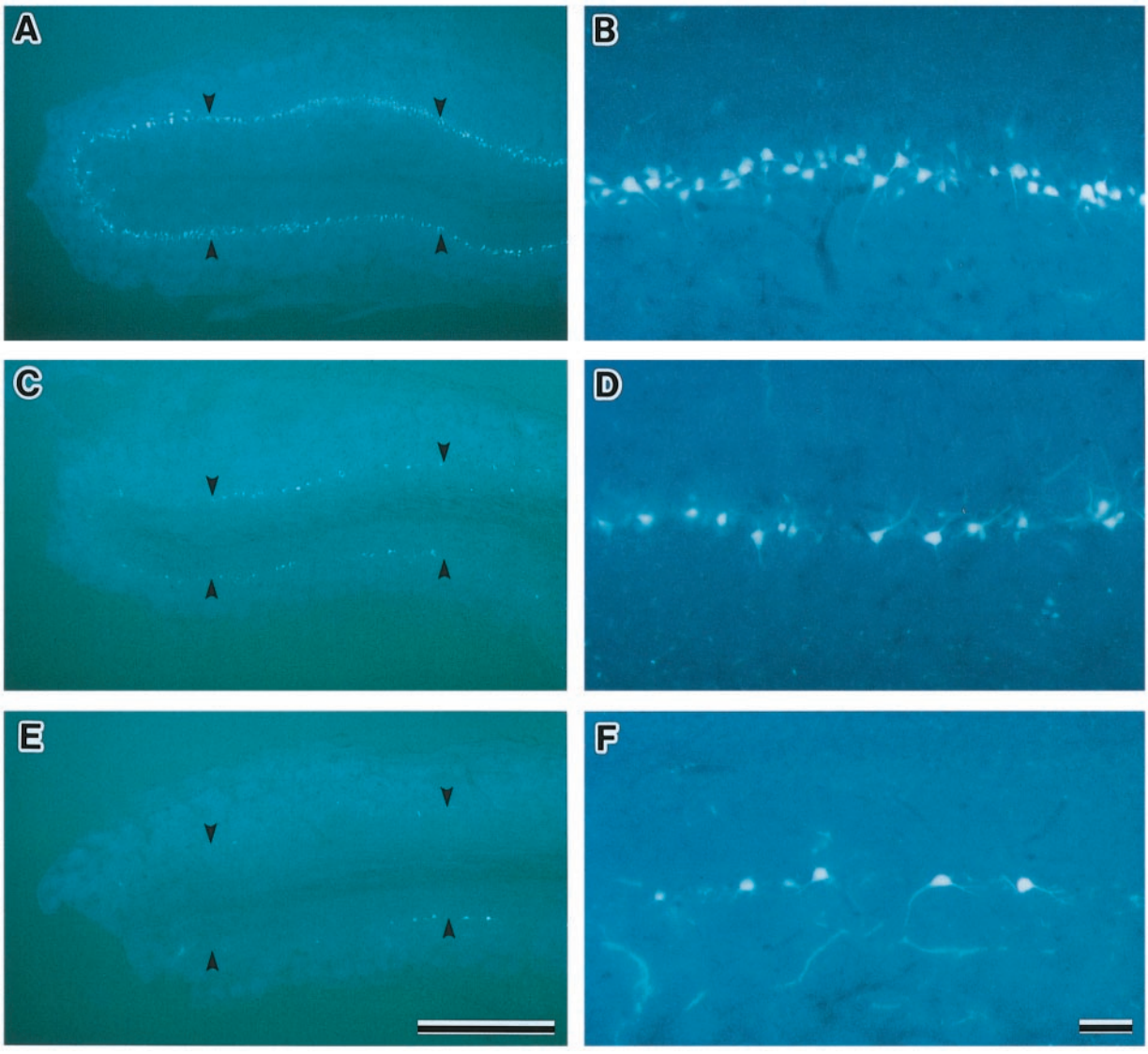

Figure 3 FG-positive mitral cells with fiber connections to the olfactory cortex. **(A, B)** Control rat. **(C, D)** LOT-lesioned rat with normal olfaction. **(E, F)** LOT-lesioned rat with loss of olfaction. Note the gradual decrease in the number of FG-positive mitral cells from control to anosmia. Each arrowhead (A, C, E) points to the mitral cell layer. Scale bars: (A, C, E) = 1 mm; (B, D, F) = 100 μm.

cells per unit length of the mitral cell layer were 47–54 (50 ± 3) cells/mm in the control rats, 10–46 (21 ± 13) cells/mm in the LOT-lesioned rats with normal olfaction and 0–11 (3 ± 4) cells/mm in the LOT-lesioned rats with anosmia (Figure 4). The Mann–Whitney *U* test showed statistically significant differences between the control and normal groups ($P = 0.0027$), control and anosmic groups ($P = 0.0003$), and normal and anosmic groups ($P = 0.0002$).

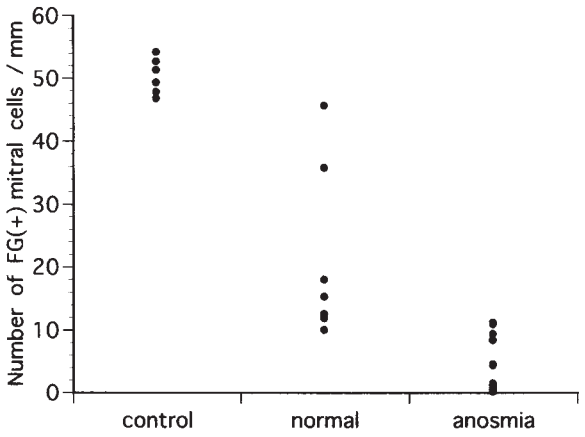

Figure 4 Average numbers of FG-positive mitral cells per unit length of the mitral cell layer. By the olfactory discrimination test, the animals are divided into three groups: control rats ($n = 6$), LOT-lesioned rats with normal olfaction ($n = 7$) and LOT-lesioned rats with anosmia ($n = 18$). Each dot represents one animal, but many dots are overlapping in anosmic animals. Note that the threshold number of FG-positive mitral cells is around one-fifth (10 cells/mm) of the control value (50 cells/mm).

Discussion

Some methodological problems had to be overcome to pursue this study. One was how to approach the LOT. Since the LOT is located ventrolaterally to the retrobulbar olfactory stria, it is difficult to reach it from the vertical direction that is generally used in stereotaxic brain experiments. In this study, we could access the LOT from the lateral direction, which is often accepted in skull base surgery. Consequently the LOT was clearly identified as a white continuous band connecting the olfactory bulb to the olfactory cortex. Among the many central sensory pathways of the brain, the LOT has the advantage that we can access it and make a well-localized lesion on it without involving the surrounding tissues. Although the anosmic rats generally had larger LOT lesions than the rats with normal olfaction, it should be mentioned that even small LOT lesions of <1 mm diameter caused anosmia in cases where the lesions were made on the more posterior LOT, where the relatively broad band of the retrobulbar LOT gradually converges to a narrow band.

A second problem was whether we could totally label the mitral cell population by a retrograde neuronal tracer. Of many fluorescent tracers, FG turned out to be a good tracer for this purpose because it easily diffused far from an injection site to cover the whole olfactory cortex. Further, complete overlapping of FG-positive cells with PI-positive mitral cells in the control rats indicates that FG injections into the olfactory cortex successfully produced total labeling of mitral cells, which is a necessary prerequisite for measuring the quantity of olfactory projection neurons connected to the olfactory cortex. Since neuronal cell bodies of the axotomized mitral cells survived in the bulb, it is absolutely necessary to use a neuroanatomical tracer to estimate the mitral cells with fiber connections to the olfactory cortex. In the LOT-lesioned cases, neuronal fiber tracing gave objective scores for the degree of LOT lesioning by visualizing healthy mitral cells that transported the fluorescent tracer retrogradely from the olfactory cortex.

The present study showed that in anosmic animals there were <11 (0–11) healthy mitral cells/mm, and that in animals with normal olfaction there were >10 (10–46) cells/mm. Therefore, it is concluded that the essential mitral population required for olfactory function is ~20% of the control value (50 ± 3 cells/mm), a threshold value for olfaction. Recently, Lu and Slotnick (Lu and Slotnick, 1998) examined olfactory function in animals with varying degrees of bulbectomy. In this connection, it should be mentioned here that removal of the anterior olfactory bulb often causes damage to the olfactory nerves connecting to the posterior bulb, and that removal of the posterior olfactory bulb often causes damage to the axon fibers derived from the mitral cells in the anterior bulb. Thus the volume of intact bulb with partial bulbectomy is not necessarily proportional to the number of intact mitral cells that relay olfactory information from the olfactory epithelium to the olfactory cortex. To know the neuronal number of intact mitral cells precisely, our experiments have an advantage that lesions were made only on the central olfactory pathway (LOT), leaving both the olfactory epithelium and the olfactory bulb totally intact. However, it is interesting that our results turned out to match well with those of the previous study (Lu and Slotnick, 1998) reporting that bulbectomized rats with bulbar savings scores of <21% had deficits in odor detection or discrimination tasks. Considering that the total number of mitral cells in each olfactory bulb has been reported to range between 45 000 and 75 000 in the rat (Meisami and Safari, 1981; Rosselli-Austin and Williams, 1990; Bonthius *et al*., 1992; Royet *et al*., 1998), the absolute number of olfactory projection neurons required to discriminate between cycloheximide solution and water can be simply calculated to be 9000–15 000 in the unilateral olfactory system. When the bilateral olfactory system is intact, normal olfactory function can be preserved with <20% of the olfactory projection neurons in terms of the ability to discriminate cycloheximide solution from water. Furthermore, it should be mentioned here that the olfactory function examined in the present study is based upon the fact that the anterior part of the

olfactory cortex (piriform cortex) located between the bulbar posterior end and the lesioned LOT is intact.

In the past decade, there have been important advances in olfactory cognition. Each type of odorant receptor expressed in olfactory receptor neurons has been reported to correspond to a single specific bulbar glomerulus (Buck and Axel, 1991; Mombaerts *et al*., 1996). Rubin and Katz (Rubin and Katz, 1999) have revealed that odorants are encoded by distinct spatial patterns of bulbar glomerular activation. These studies strongly suggest the existence of distinct topographies between olfactory receptor neurons and bulbar glomeruli, depending on odorants. Although we proposed that the essential neuronal population in the central olfactory pathway is about one-fifth of the total population, the exact proportion should be determined for different odors. An important question about the existence of receptor-related topographic pathways in the higher olfactory centers should be explored in the near future.

Many scientists are paying great attention to stem cells of the brain for the realistic purpose of treating brain disorders (Reynolds and Weiss, 1992; McKay, 1997; Roy *et al*., 2000). However, there are no data available about the number of essential neurons for the functional repair by transplanted brain stem cells. The present results might provide fundamental data on a presumed quantity of replaceable neurons required for the functional recovery of the injured brain.

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