

Long-term Recovery of the Rat Vomeronasal System after Nerve Transection

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Summary. The vomeronasal system in adult animals has the capacity to produce new receptor cells continuously. When the vomeronasal nerve bundles in rodents are transected, or the accessory olfactory bulb (AOB) is removed, vomeronasal receptor cells degenerate and immediately start to regenerate receptor cells. To investigate in further detail the changes to the vomeronasal system during the long recovery period following nerve transection, we conducted an immunocytochemical analysis of the AOB using anti-NCAM (neural cell adhesion molecule), anti-Gi2 α , and anti-synaptophysin (SYN) antibodies, together with electron microscopy of the vomeronasal organ (VNO) in rats. On day 200 after nerve transection, NCAM, Gi2 α and SYN immunoreactivity (ir) was observed within the vomeronasal nerve layer (VNL) and/or glomerular layer (GL) in the AOB of four of ten rats, but the immunoreactive areas were smaller than those in control sections (positive group). The remaining six rats showed no ir for NCAM, Gi2 α , or SYN within the VNL and/or GL in the AOB (negative group). The percentage of receptor cells with microvilli relative to the total number of receptor cells contacting the luminal surface of the VNO was calculated, showing no difference evident when comparing the positive and negative groups; that is, the microvilli of receptor cells were observed whether or not there were connections between the VNO and the AOB. These results suggest that regeneration of the VNO and AOB occurs independently.

Key words—Accessory olfactory bulb, Immunocytochemistry, Rat, Vomeronasal organ.

INTRODUCTION

The olfactory organ is located in the caudal part of the nasal cavity. Their constituent cells contain apical dendrites that are covered with cilia extending into the nasal cavity where they come in contact with volatile substances. The vomeronasal organ (VNO) is located within a bony capsule along the nasal septum. Vomeronasal receptor cells occupy about three-fourths of the basal sensory epithelium. At the surface of this epithelium, dendrites of vomeronasal receptor cells are covered with microvilli that extend into the lumen. Axons from both olfactory and vomeronasal receptor cells pass through the cribriform plate and project to different regions of the olfactory bulb. Axons from the olfactory receptor cells terminate in the main olfactory bulb (MOB) and those from the vomeronasal receptor cells project to the accessory olfactory bulb (AOB). Both the olfactory and vomeronasal systems have the capacity to regenerate new receptor cells throughout an animal's life. In the olfactory system, removal of the olfactory bulb or olfactory nerve transection leads to the regeneration of olfactory receptor cells and reestablishment of projections to the main olfactory bulb^{1,2}. The functional recovery of receptor cells and reestablishment of their central connections occur after a recovery period of 40 days³.

In the vomeronasal system, the continuous regeneration of receptor cells also occurs in both young and adult animals. When the vomeronasal nerve bundles are transected or the olfactory bulb is removed, vomeronasal receptor cells regenerate after degener-

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ation⁴⁻⁹). Although the newly regenerated receptor cells have the capacity to reestablish connections with the AOB, the reconnection rate of the vomeronasal system is lower than that of the olfactory system^{2,10}). Several studies examining immunocytochemical changes in the olfactory system during regeneration have been reported¹¹⁻¹⁴), though there have been only a few studies in the vomeronasal system on immunocytochemical changes in the epithelium following nerve transection. Recently, Matsuo-ka et al.¹⁵) revealed that, although the olfactory epithelium showed morphological recovery on day 60 after nerve transection, the recovery of vomeronasal receptor cells was not evident by day 60. Ichikawa¹⁰) showed that axons of vomeronasal receptor cells could reach the AOB after day 120, but the period at which the VNO attains morphological and functional maturation remains unknown. To investigate whether the vomeronasal system requires a longer time to recover and to determine the relation between the connection of receptor cells to the AOB and their maturational state, we conducted an immunocytochemical study of the AOB using three antibodies, namely anti-NCAM, anti-Gi2 α , and anti-synaptophysin (SYN) antibodies, 200 days following nerve transection. As NCAM is expressed in most immature and mature neurons¹⁶), this was used as a marker for sensory neurons. Gi2 α is expressed in the apical vomeronasal receptor cells in the vomeronasal epithelium and rostral halves of the AOB in mature animals¹⁷), making it a suitable marker for the vomeronasal system. Synaptophysin (SYN) is expressed in the synaptic glomerular layer¹⁸), and so was used as a marker for synaptic connections. In addition, we performed electron microscopy to observe the degree of maturation of the vomeronasal sensory epithelium.

MATERIALS AND METHODS

Animals

Male adult Sprague-Dawley (SD) rats having a body weight of between 280 to 316 g were housed in a temperature-controlled room under a 12 hr/12 hr light/dark cycle. The animals were kept in acrylic cages with woodchip bedding and had unlimited access to the usual laboratory chow and water. All experimental procedures employed were approved by the Institutional Animal Care and Ethics Committee of the Tokyo Metropolitan Institute for Neuroscience.

Surgery

The rats were anesthetized with ketamine hydrochloride (85 mg/kg) and xylazine (10 mg/kg) prior to surgery. The frontal bone covering the left olfactory bulb was removed and a custom-made Teflon blade was used to cut both the vomeronasal and olfactory nerve fibers passing between the cribriform plate and the left olfactory bulb⁵). The vomeronasal and olfactory nerves on the right side were left intact and served as the unoperated control. Following surgery, the animals were monitored carefully until they recovered from anesthesia and were then returned to their own cages.

Immunocytochemistry and electron microscopy

The animals (N=10) were deeply anesthetized with sodium pentobarbital and perfused with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). For immunocytochemical examination, the AOB was removed and fixed in the same fixative for 24 hr at 4°C. The tissue was cut into 30 μ m-thick sections using a freezing microtome and sections were incubated for 24-48 hr at 37°C with one of the following antibodies: anti-NCAM antibody (Sigma) diluted to 1 : 100, anti-Gi2 α antibody (Wako) diluted to 1 : 500, and anti-SYN (Böhringer-Manheim) diluted to 1 : 500. After washing in phosphate-buffered saline (PBS) for 30 min, the sections were incubated with anti-mouse Ig or anti-rabbit Ig biotinylated antibody diluted to 1 : 200 for 1 hr at 37°C, then washed in PBS for 30 min, and incubated with streptavidin labeled with a fluorescent dye. The sections were washed in PBS for 30 min, coverslipped with a water-based mounting medium (PermaFluor, LIPSHAW), and examined with a fluorescence microscope (Axiophot, Zeiss). For electron-microscopic examination, the VNO was removed and fixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. It was cut into sections of approximately 1.0 mm thickness with a razor blade and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in ethanol, and embedded in plastic resin (Quetol 812). Ultrathin sections were cut with a microtome (MT 7000, MRC). After being stained with uranyl acetate and lead citrate, the sections were examined with an electron microscope (Hitachi, model H-7500). Micrographs of the luminal surface of the VNO were obtained from ten animals, and the numbers of receptor cells both with and without microvilli were counted. The percentage of receptor cells with more than one microvillus with respect to the total number of receptor cells was calculated.

RESULTS

Immunocytochemistry

In control sections of the AOB, strong NCAM immunoreactivity (ir) was observed within the vomeronasal nerve layer (VNL) and glomerular layer (GL) whereas no ir was observed within the mitral/tufted cell layer (MTL), olfactory tract layer (OTL) or granule cell layer (GRL) (Fig. 1 A). On day 200 following nerve transection, NCAM-ir could not be observed in the AOB of six of the ten rats studied (Fig. 1 B). The other four animals showed NCAM-ir within the VNL and GL (Fig. 1 C). The reactive area was mainly in the rostral half of the AOB, but some animals showed reactivity in the caudal half of the AOB. In control sections of the AOB, a strong Gi 2 α -ir was observed within the rostral halves of the VNL and GL (Fig. 1 D). The MTL, OTL, and GRL did not show Gi 2 α -ir. On day 200, Gi 2 α -ir could not

be observed in the AOB of six out of ten rats (Fig. 1 E). The other four animals showed Gi 2 α -ir in the rostral halves of the VNL and GL (Fig. 1 F). In control sections of the AOB, strong SYN-ir was observed within the GL, MTL, and GRL. The VNL and OTL did not show SYN-ir (Fig. 1 G). On day 200, SYN-ir was observed within the MTL and GRL of the AOB of six of the ten rats studied (Fig. 1 H). The other four animals showed SYN-ir within the GL, MTL, and GRL of the AOB (Figs. 1 I). The four animals making up the positive group were always the same four individuals, regardless of which antibody results were used to group them. The areas on the nerve transected side showing NCAM-ir, Gi 2 α -ir and SYN-ir were smaller than on the control side in all experimental animals.

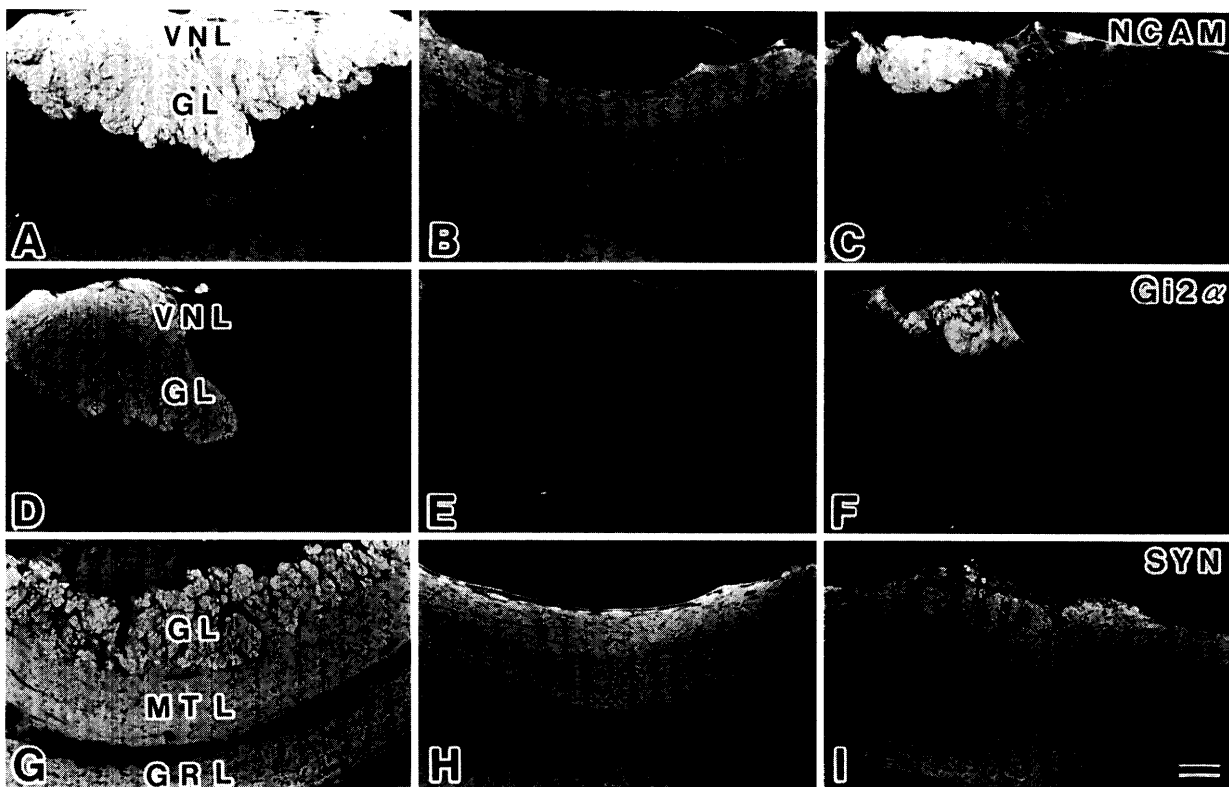


Fig. 1. Immunoreactivity in the accessory olfactory bulb (AOB) 200 days after nerve transection. Control section shows NCAM-ir in the vomeronasal nerve layer (VNL) and glomerular layer (GL), Gi 2 α -ir in rostral halves of the VNL and GL, and synaptophysin (SYN)-ir in the GL, MTL and GRL (Figs. 1A, D and G). Six of the ten rats show a negative reaction to antibodies against NCAM and Gi α in all layers of the AOB (Figs. 1B and E), and SYN in VNL and GL (Fig. 1H). The other four rats show NCAM-ir and Gi α -ir within the VNL and GL, and SYN-ir within the GL, MTL, and GRL (Figs. 1C, F and I). Scale Bar, 200 μ m.

Electron microscopy

Electron-microscopic examination was performed for both the control side (right) and operated side (left) of the VNO 200 days after nerve transection. In the control sections, receptor cells were observed to be covered with thin microvilli. Close to their base, the receptor cell microvilli were thick, but appeared tapered and thin toward the tips. The diameter of the receptor cell microvilli was about 45 nm. In contrast, supporting cells had uniformly thick microvilli. The diameter of the supporting cell microvilli was about 90 nm. Numerous receptor and supporting cell microvilli were observed to cover the luminal surface of the cells in control sections (Fig. 2 A). On day 200 after nerve transection, considerable variability in the extent of recovery was observed in the sensory epithelium (Figs. 2 B and 2 C). In some regions of the sensory epithelium, receptor cell microvilli were still scanty (Fig. 2 B), whereas in other regions, numerous receptor cell microvilli covered the luminal surface of the vomeronasal sensory epithelium. These receptor cells existed side by side (Fig. 2 C). Degenerating receptor cells were seen in the region of the epithelium that showed incomplete recovery of the microvilli (asterisk in Fig. 2 B). The microvilli covering the supporting cells were still observed on the surface of the epithelium. Knob-like structures were seen protruding into the lumen from the dendritic ends of a few receptor cells; these structures also contained centrioles (data not shown).

The ratio of receptor cells with microvilli in the VNO

Four of the ten rats showed NCAM-ir, Gi 2 α -ir and SYN-ir within the VNL and/or GL in the AOB. These four were called the positive group. The other six animals showed no ir to these antibodies within the VNL and/or GL in the AOB and so were called the negative group. To compare the degree of maturation of both groups of animals, we counted the number of receptor cells exhibiting more than one microvillus in each group, and expressed these numbers as percentages of the mean of the total number of receptor cells for each condition. On the control (unoperated) side, all cells exhibited more than one microvillus, whether or not the animal was from the positive or negative group. On the nerve-transected side, the mean percentage of cells exhibiting more than one microvillus was 36.1% for the positive group, while that for the negative group was 29.9%; this difference did not reach statistical significance (Table 1, Mann-Whitney's U-test).

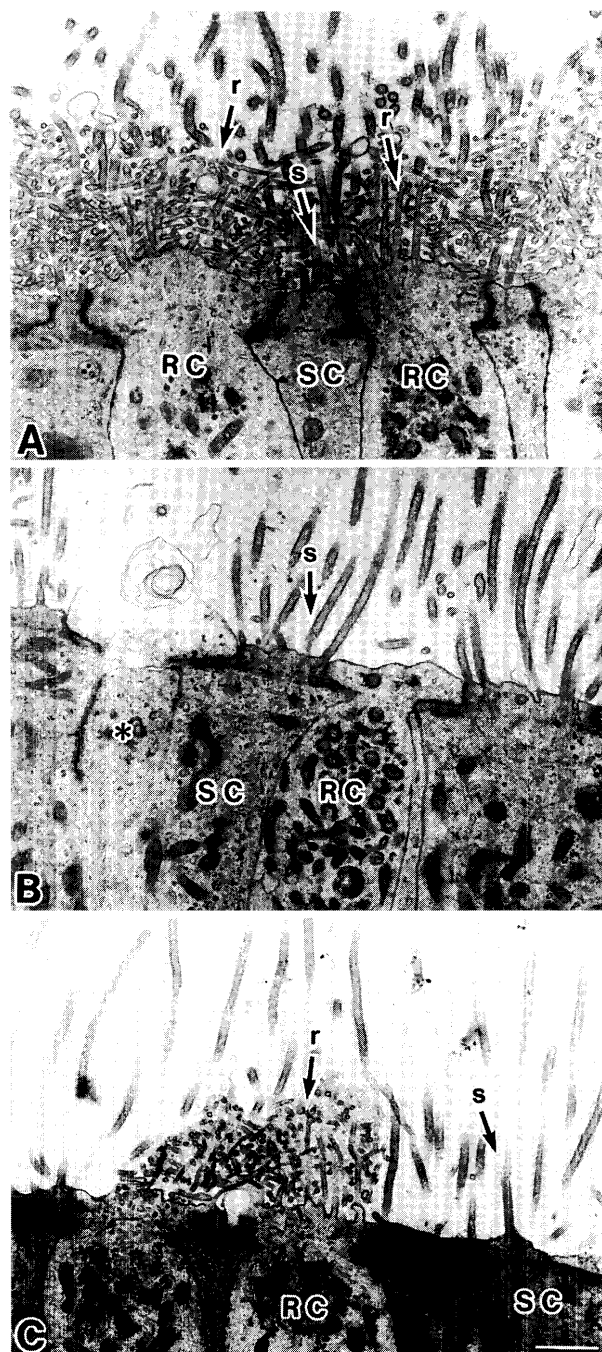


Fig. 2. Morphological changes in the vomeronasal epithelium during the regeneration of receptor cells. **A.** Normal morphology of the epithelial surface with microvilli on two types of cells. Receptor cell microvilli (r) are thin and tapered while the supporting cell microvilli (s) are thicker and of more uniform diameter. **B** and **C.** On day 200, some regions of the sensory epithelium had few microvilli (**Fig. 2B**) whereas the other regions showed numerous mature microvilli (**Fig. 2C**). A degenerating receptor cell (*asterisk*) is shown in **Fig. 2B**. RC, receptor cell; SC, supporting cell. Scale Bar, 1 μ m.

Table 1. The percentage of receptor cells with microvilli with respect to the total number of receptor cells in the VNO

Group	Number of animals	Percentage (% \pm S.E.M)
Positive	4	36.1 \pm 8.7
Negative	6	29.9 \pm 6.8

DISCUSSION

One of the aims of the present study was to investigate the relationship between the recovery of projections to the AOB and the maturational state of vomeronasal receptor cells. Animals belonging to the positive groups showed NCAM-ir, Gi 2 α -ir, and SYN-ir within the VNL and/or GL in the AOB, and those in the negative group showed no ir to the three antibodies. These results suggest that the vomeronasal receptor cells may have differentiated 200 days after nerve transection and may have the capacity to form new synaptic connections with the mitral/tufted cells within the AOB. A previous morphological study showed that 30% of the operated animals recover projections to the AOB after nerve transection¹⁰. Our result is consistent with this study.

In addition, we calculated the percentage of receptor cells having more than one microvillus in animals from each group so that we could investigate the maturational level of the vomeronasal receptor cells. Microvilli are thought to be important structures with respect to pheromone receptor function, as they possess pheromone receptors¹⁹. There were no differences in percentages of receptor cells having more than one microvillus and those not having more than one microvillus, when comparing the positive and negative groups. In the olfactory system, ciliary growth from knob-like structures occurs as a result of the formation of connections between receptor cells and the MOB, and this connection helps maintain a cell until it matures^{20,21,22}. These studies revealed that the axonal projections to the target were required for the maturation of the olfactory receptor cells. In the vomeronasal system, the present study showed that the microvilli of the receptor cells could be observed regardless of whether there were connections between the VNO and the AOB. This observation suggests that the AOB is unnecessary for the morphological maturation of the vomeronasal receptor cells after vomeronasal nerve transection, but it remains possible that projections to the target

might play some role in functional maturation, that is, through the expression of pheromone receptors and second messengers.

Several studies on regeneration of the olfactory system have shown that the system has the capacity to produce new cells, form connections with its target (MOB), and recover its function^{3,23,24}. On the other hand, the reconnection rate of vomeronasal receptor cells to the AOB is very low compared with that of the olfactory receptor cells¹⁰. To explain this, we should consider the location of the olfactory and vomeronasal organs. The olfactory organ is located in the caudal part of the nasal cavity and its rostral end is directly exposed to the external environment of the animal's body. This structure helps in detecting most vaporized chemicals. The vomeronasal organ (VNO) is located within a bony capsule along the nasal septum and its rostral end opens to the nasal cavity. In the vomeronasal system, special methods for pheromone detection, such as the pumping mechanism, head-bobbing, and flehmen, are employed because of the organ's location and the narrow structure of the vomeronasal lumen^{25,26,27}. In intact animals, it is considered that the olfactory system has developed a tolerance to toxic chemicals and recovers from damage to the olfactory epithelium because neurotoxic substances can easily access the olfactory epithelium. On the other hand, the inhalation of neurotoxic substances into the vomeronasal sensory epithelium rarely happens because of the latter's location. Such differences may affect the occurrence of neurogenesis and the recovery rate of these two organs following the loss of their stimuli. Further studies are needed to examine the influence of trophic factors in the environment on the olfactory system or the vomeronasal system during regeneration.

The present study considers the influence of the longer recovery period and the importance of the target tissue for the maturation of the vomeronasal system. We conducted immunocytochemistry of the AOB and electron microscopy of the vomeronasal organ on day 200 after nerve transection. Even though a longer recovery period was allowed in these studies than previously, the organs had yet to reach a complete morphological maturational state. Microvilli, a symbol of mature vomeronasal receptor cells, were observed whether or not there were any connections between the VNO and the AOB. These results suggest that regeneration of the VNO and the AOB occurs independently.

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