Morphological Analysis of Olfactory Receptor Cells Using Whole-Mount Preparations of the Rat Nasal Mucosa

Yoshinao HOSAKA, Haruko YANASE and Toshihiko IWANAGA

Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo, Hokkaido 060–0818, Japan

(Received 5 February 1997/Accepted 3 April 1998)

ABSTRACT. The distribution and entire shape of olfactory receptor cells were investigated by means of whole-mount preparations of the nasal mucosa. Whole mucosa isolated from the nasal septum of rats was processed, as "a free-floating section", and examined by the avidin-biotin complex (ABC) method using antisera against protein gene product 9.5 (PGP 9.5) and calbindin. Essentially all receptor cells were immunolabeled with the PGP 9.5 antiserum, but only half of PGP 9.5-immunoreactive cells were calbindin-immunoreactive. In the immunostaining of whole-mount preparations, pretreatment of tissues by freeze-thawing and dipping in ethanol and xylene greatly improved the permeability of antibodies. Overview of the nasal septum showed that the dorsal and ventral portions of the rostral olfactory area extended deeply into the respiratory area, making a "semi-lunar" shape. The boundary between the two areas was clearly demarcated, although several receptor cells were scattered in the respiratory area near the boundary. Observation at higher magnification clearly demonstrated that several axons derived from perikarya gathered to form nerve bundles showing a dendritic pattern. Proximal axons close to perikarya displayed beaded structures with intense immunoreactivity. They were electron-microscopically identified as swollen portions of axons which might be formed in association with the axonal flow. The present study showed that whole-mount preparation of the nasal mucosa for immunohistochemistry is a useful tool to analyze the morphology of olfactory receptor cells and axons. — KEY WORDS: calbindin, immunohistochemistry, olfactory receptor cell, PGP 9.5, whole-mount preparation.

J. Vet. Med. Sci. 60(8): 897–904, 1998

Immunohistochemistry using neuron-specific proteins is widely employed at present as a reliable staining method for neurons instead of classical silver impregnation methods. Several neuron-specific proteins, such as neuron-specific enolase (NSE) and neurofilament protein (NFP), are shared by sensory cells and endocrine cells [10]. For immunostaining of olfactory receptor cells, NSE, NFP and olfactory marker protein (OMP) have been used as markers of the cells. These proteins, however, have some shortcomings for immunostaining; antisera against OMP are very difficult to get due to the limited supply, NSE is usually weak in antigenicity and NFP antisera can stain only small populations of olfactory receptor cells. Furthermore, these antisera are not able to stain the entire length of the receptor cell from the tip of the dendrite to the axon terminal [10, 20].

Protein gene product 9.5 (PGP 9.5), which was originally identified in extracts of the human brain, abundantly exists in the cytosol of essentially all neurons and is recognized as a useful marker for neurons [25]. Iwanaga *et al.* [11] demonstrated that PGP 9.5 was also contained in sensory paraneurons and that in the nasal mucosa only receptor cells showed PGP 9.5 immunoreactivity. On the other hand, calbindin is a Ca^{2+}-binding protein isolated from the small intestine, kidney and cerebellum. Immunohistochemistry using calbindin antisera revealed that Purkinje cells were selectively stained in the cerebellum [30, 32] and that this protein was also shared by some sensory paraneurons, including olfactory receptor cells [1, 9, 12, 13].

Morphological studies of olfactory receptor cells have been done mainly by using thin tissue sections. Even if we can specifically immunolabel the receptor cells, it is difficult to visualize their distribution and morphology throughout the nasal mucosa.

Whole-mount preparation has the advantage of making it possible to observe wide areas and the entire shape of a structure, even three-dimensionally. Its usefulness has been shown in observation of nerve fibers and lymphatic vessels distributed in membranous tissues such as the iris, urethra, intestine and tracheal mucosa [4, 21, 22, 26]. In the present study, immunohistochemical staining using PGP 9.5 and calbindin antisera was applied to whole-mount preparations of the nasal mucosa to demonstrate the detailed distribution and entire shape of olfactory receptor cells including the dendrite and axon. Furthermore, electron microscopic observation revealed the nature of beaded structures found on proximal axons of receptor cells.

MATERIALS AND METHODS

Immunohistochemistry: Thirty-four male Wistar rats, weighing 200–300 g, were used in this study. The animals were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 m*l*/kg body weight) and perfused through the ascending aorta with physiological saline and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The nasal septum was removed carefully, immersed in the fixative for 6 hr, and immersed in 30% sucrose dissolved in the phosphate buffer overnight at 4°C.

For frozen sections, tissues embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) were quickly frozen in liquid nitrogen. Frozen sections were cut at a thickness of 20 μm and examined by the avidin-biotin complex (ABC) method. After pretreatment with normal goat serum, the sections were incubated in rabbit anti-human PGP 9.5 serum (1:6,000; Ultraclone, England) or rabbit anti-rat calbindin serum (1:10,000; provided by Dr. M. Watanabe, School of Medicine, Hokkaido Univ., Japan). The antigen-antibody reaction was visualized by biotin-labeled goat anti-rabbit immunoglobulins and peroxidase-streptavidin complex (Histofine; Nichirei, Tokyo, Japan). An enzyme reaction was developed with a mixture of diaminobenzidine (DAB; 0.04%) and H_2O_2 (0.002%) in 0.05 M Tris-HCl buffer, pH 7.6.

Whole-mount preparations were obtained by peeling the mucosa from the nasal septum, and processed for "free-floating" immunostaining essentially by the procedure described above. Incubation time was two or three times longer than that for the frozen sections. Stained preparations were mounted on gelatin-coated glass slides (the epithelium was on top) and dried on a hot plate. For improving permeability of antibodies, tissues were frozen in liquid nitrogen, thawed at room temperature, and passed through a graded series of ethanol and xylene before immunostaining. All phosphate-buffered saline (PBS; 0.1 M, pH 7.2) used for washing and diluting antisera contained 0.3% Triton X-100. Tissues were stirred by a rotor throughout the staining and washing process. Furthermore, to analyze the axons of receptor cells, the apical layer of the olfactory epithelium was mechanically removed from the whole-mount preparation using the blunt edge of razor blades.

Conventional electron microscopy: Seven male Wistar rats were perfused with physiological saline, followed by 2.5% glutaraldehyde in 0.06 M sodium cacodylate, pH 7.4. The tissues were dissected into small pieces and fixed in the same fixative for 2 hr. After post-fixation with 1% OsO_4 for 1.5 hr, they were dehydrated through a graded series of ethanol and embedded in Quetol 812 according to the standard procedure. Ultrathin sections, 110 nm in thickness, were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (TEM; JEM-1210, JEOL, Japan).

Immunoelectron microscopy: Frozen sections, 20 μm in thickness, were prepared from the paraformaldehyde-fixed nasal mucosa, and subjected to the ABC method using the PGP 9.5 antiserum (1:6,000 in dilution) as mentioned above. The pretreatment with Triton X-100-containing PBS was omitted in this staining. After DAB reaction, the stained sections were post-fixed with 1% OsO_4 for 30 min, dehydrated in an ethanol series and embedded in Quetol 812. Ultrathin sections, 110 nm in thickness, were prepared, lightly stained with lead citrate and examined with the TEM.

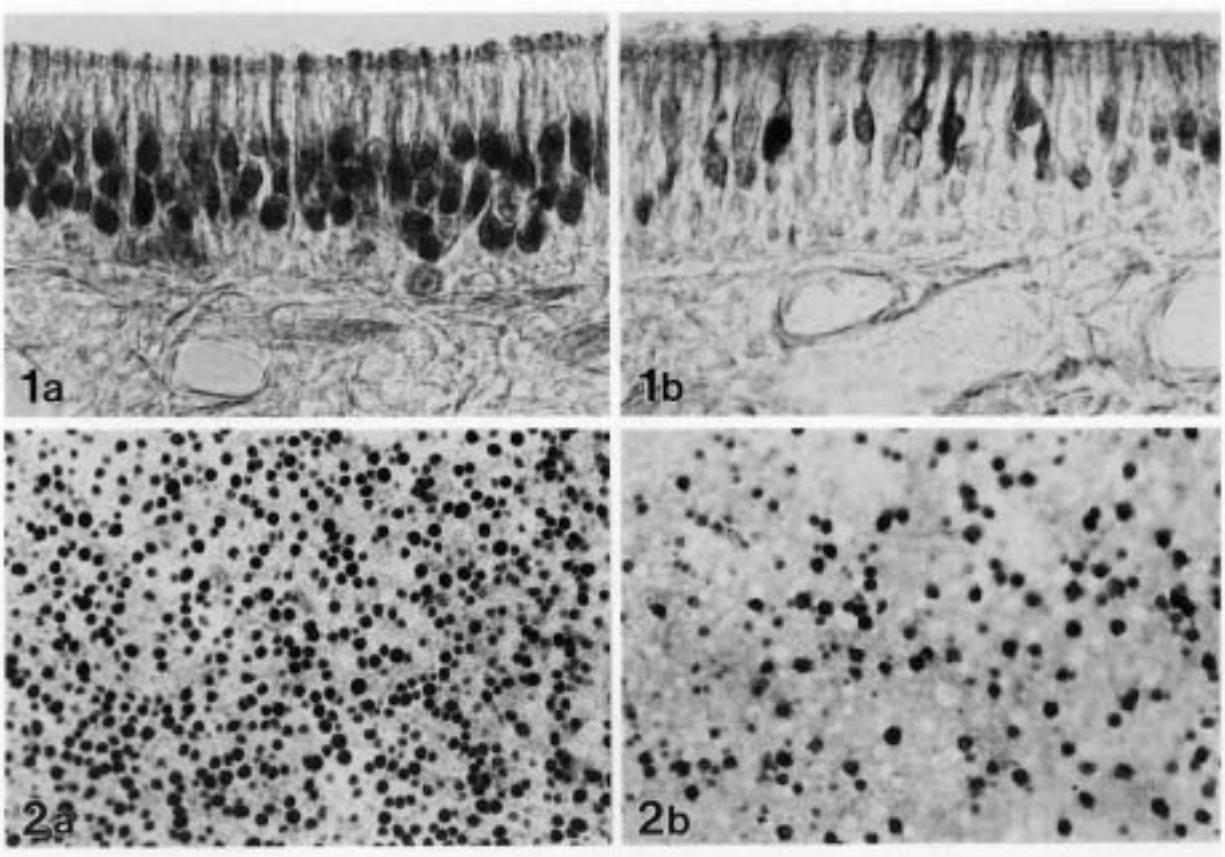
For more detailed observation of immunoreactive structures, immunostained sections were processed for immunogold labeling with silver enhancement [3, 22]. Before incubation with the PGP 9.5 antiserum, frozen sections were immersed for 5 min in 50 mM glycine in PBS to quench aldehyde. After the sections were incubated with a colloidal gold-conjugated anti-rabbit IgG (NANOGOLD; Ø 1.4 nm, NANOPROBES, U.S.A.) diluted 1:600 overnight at 4°C, they were fixed by 1% glutaraldehyde for 5 min, and immersed in 0.1 M acetic acid buffer (pH 7.0) for 10 min. The sections were then enhanced with a silver enhancement kit (HQ SILVER; NANOPROBES, U.S.A.) for 8 min in a dark room. The enhanced sections were post-fixed with 1% OsO_4 for 30 min, dehydrated in an ethanol series and embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a TEM.

RESULTS

Observation of serial frozen sections alternatively stained using PGP 9.5 and calbindin antisera showed that immunoreactivity for PGP 9.5 was found in essentially all olfactory receptor cells, whereas immunoreactivity for calbindin was localized in only half of the cells (Fig. 1). No significant difference on the shape and distribution throughout the olfactory mucosa was found between PGP 9.5- and calbindin-immunoreactive receptor cells. The restricted immunoreactivity with the calbindin antiserum was also found at the level of nerve bundles in the lamina propria. More than half of nerve fibers in nerve bundles running in the subepithelial and upper regions of the lamina propria were immunonegative for calbindin. All nerve fibers in thick bundles located in the deeper lamina propria and derived from the trigeminal nerve showed strong immunoreactivity for calbindin. In contrast, all nerve bundles were immunolabeled by the PGP 9.5 antiserum.

Observation of olfactory receptor cells in whole-mount preparations: The most serious problem for staining of whole-mount preparations was permeability of antibodies into the tissues. Pretreatment of tissues by freeze-thawing and passage through a graded series of ethanol and xylene greatly improved the permeability of antibodies, resulting in good staining results. In the immunostaining of whole-mount preparations using two antisera, a similar staining result was obtained on the distribution and shape of receptor cells, although PGP 9.5-immunoreactive cells were more numerous than calbindin-immunoreactive cells (Fig. 2). Therefore, only PGP 9.5 antiserum was used in the following immunostaining.

Using whole-mount preparations made it possible to get a bird's eye view of the distribution of receptor cells. The dorsal and ventral portions of the rostral olfactory area extended deeply into the respiratory area, making a "semi-lunar" shape (Fig. 3). Although the boundary between the two areas was clearly demarcated, several receptor cells were scattered in the respiratory area near the boundary (Fig. 4). There was an exclave of grouped olfactory cells, corresponding to the septal organ of Masera [23], in the ventral portion of the respiratory area (Fig. 3) .



Figs. 1 and 2. Immunohistochemistry of olfactory receptor cells using frozen sections (Fig. 1) and whole-mount preparations (Fig. 2). Immunoreactivity for PGP 9.5 is found in essentially all receptor cells (Figs. 1a, 2a), while less than half of them were calbindin-immunoreactive (Figs. 1b, 2b). In the whole-mount preparations (Fig. 2), pictures are focused on olfactory vesicles. × 215.

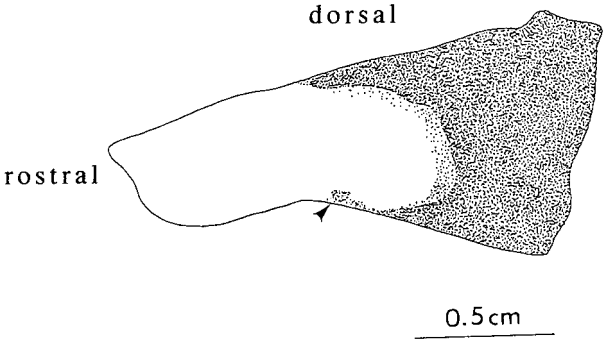


Fig. 3. A schematic diagram showing distribution of olfactory receptor cells demonstrated by observation of whole-mount preparations. The dorsal and ventral portions of the rostral olfactory area extend deeply into the respiratory area, making a "semi-lunar" shape. An exclave of grouped olfactory cells (arrowhead) corresponds to the septal organ of Masera.

In the diagonal-angle view of olfactory epithelium, the dendrites were well-observed and extended from the perikaryon toward the nasal cavity, forming the bulbous endings termed olfactory vesicles on the surface of the nasal mucosa (Fig. 5a). The dendrite shape depended on the localization of cell bodies; the perikaryon present in the apical zone of the epithelium extended a thick and comparatively straight dendrite, while basally located perikaryon possessed a thin, wavy dendrite (Fig. 5a).

To observe the axons of receptor cells, the olfactory epithelium was mechanically partially removed from whole-mount preparations. Immunostaining of these preparations clearly demonstrated detailed morphology of axons extending from perikarya; about 10 axons gathered to form nerve bundles showing a dendritic pattern (Figs. 6 and 7). Beaded structures with intense immunoreactivity were found on proximal axons close to perikarya (Fig. 5b). Globular or irregular-shaped buttons were formed along the axons at random and in some regions, several buttons lined up displaying varicosity nerves (Figs. 6 and 7). The beaded structures were further examined under an electron microscope as follows.

Electron microscopic observation of olfactory axons: Many axons derived from perikarya were observed in the basal portion of the olfactory epithelium. Some axons appeared to be isolated form and others were loosely grouped to form bundles roughly surrounded by processes of basal cells or supporting cells. Major population of axons were very thin, 0.15–0.30 μm in diameter, and contained a few microtubules (Fig. 8). This morphology corresponded

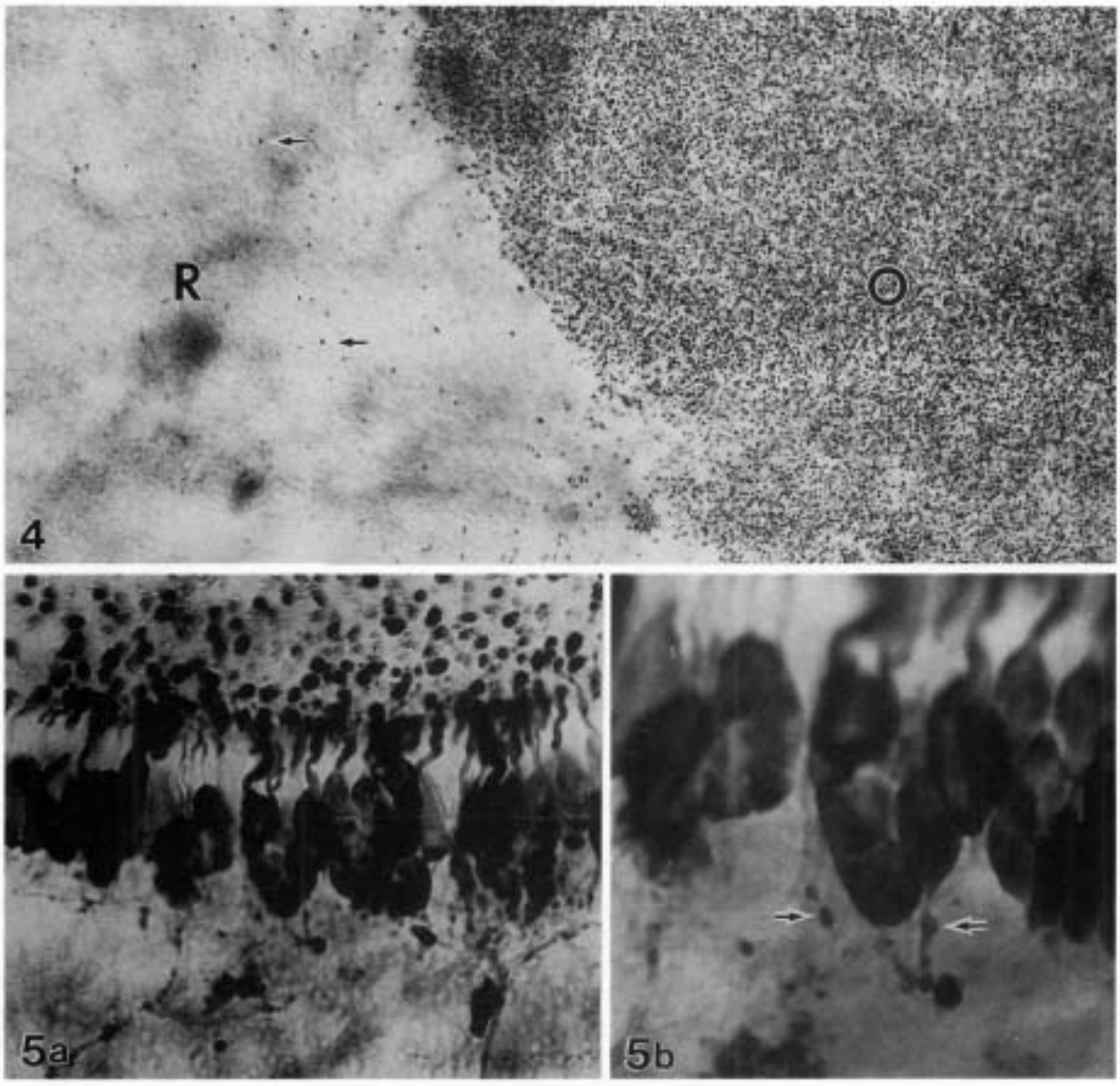
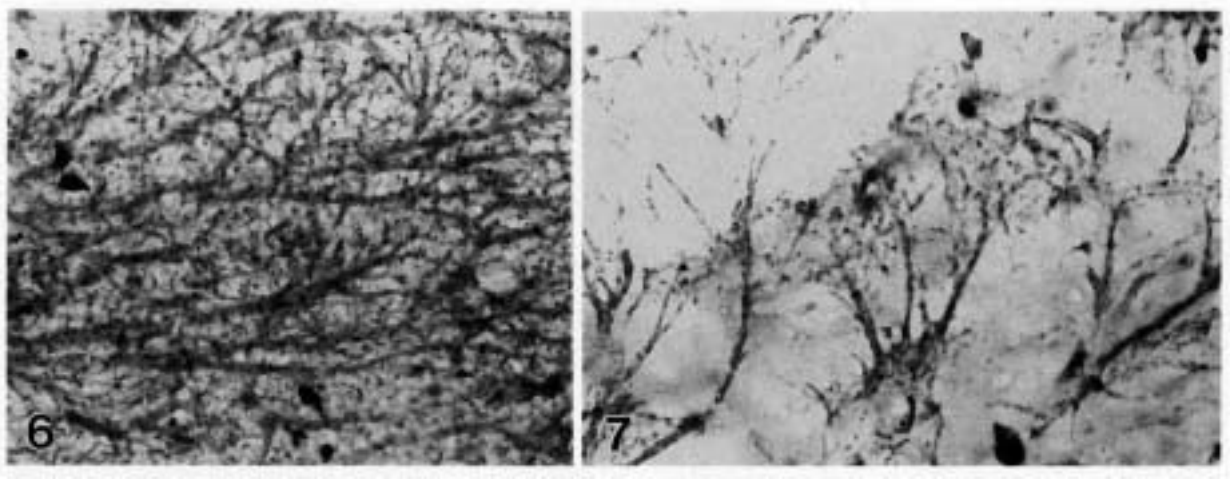


Fig. 4. A boundary area of the olfactory mucosa (O) and respiratory mucosa (R) on a whole-mount preparation stained for PGP 9.5. The boundary between the two areas is clearly demarcated, several receptor cells (arrows) being scattered in the respiratory area near the boundary. × 45.

Fig. 5. PGP 9.5-immunoreactive receptor cells on a whole-mount preparation of olfactory mucosa (diagonal-angle view). Dendrites are clearly shown to extend from the cell body of receptor cells and form olfactory vesicles on the surface of the epithelium. The perikaryon present in the apical zone of the epithelium extends a thick dendrite, while a basally located perikaryon possesses a thin and wavy dendrite. In high magnification (Fig. 5b), beaded structures of axons are seen. a × 230, b × 540.

to axons of receptor cells, which have been reported previously [5, 7, 18]. On the other hand, large, membrane-bound nerve elements with 2.0–3.0 μm in diameter were observed at the basal region of the epithelium (Fig. 9). These structures contained a few mitochondria and several synaptic vesicle-like vesicles as well as microtubules, and were held by processes or finger-like projections of basal cells.

Electron microscopic observation of tissues immunostained with the PGP 9.5 antiserum showed that large globular structures as well as thin axons were selectively immunolabeled (Fig. 10). Judging from the structural and distributional characteristics, the globular structures coincided with the large nerve elements, which contained mitochondria and synaptic vesicles. This finding was confirmed by observation of colloidal gold particle-labeled preparations (Fig. 11); gold particles indicating positive immunoreactivity were deposited on both thin and



Figs. 6 and 7. Whole-mount preparations showing proximal axons running in the basal region of the olfactory epithelium. The apical layer of the olfactory epithelium was removed in this preparation. About 10 axons with PGP 9.5 immunoreactivity gather to form nerve bundles showing a dendritic pattern. The olfactory bulb is located in the left side of Fig. 6. In higher magnification (Fig. 7), globular or irregular-shaped buttons are seen along the axons at random. Fig. 6 × 120, Fig. 7 × 280.

globular structures. Furthermore, both structures were found to be connected directly. Immunoreactive globular structures on the axons were observed regardless of whether they were over or under the basal membrane.

DISCUSSION

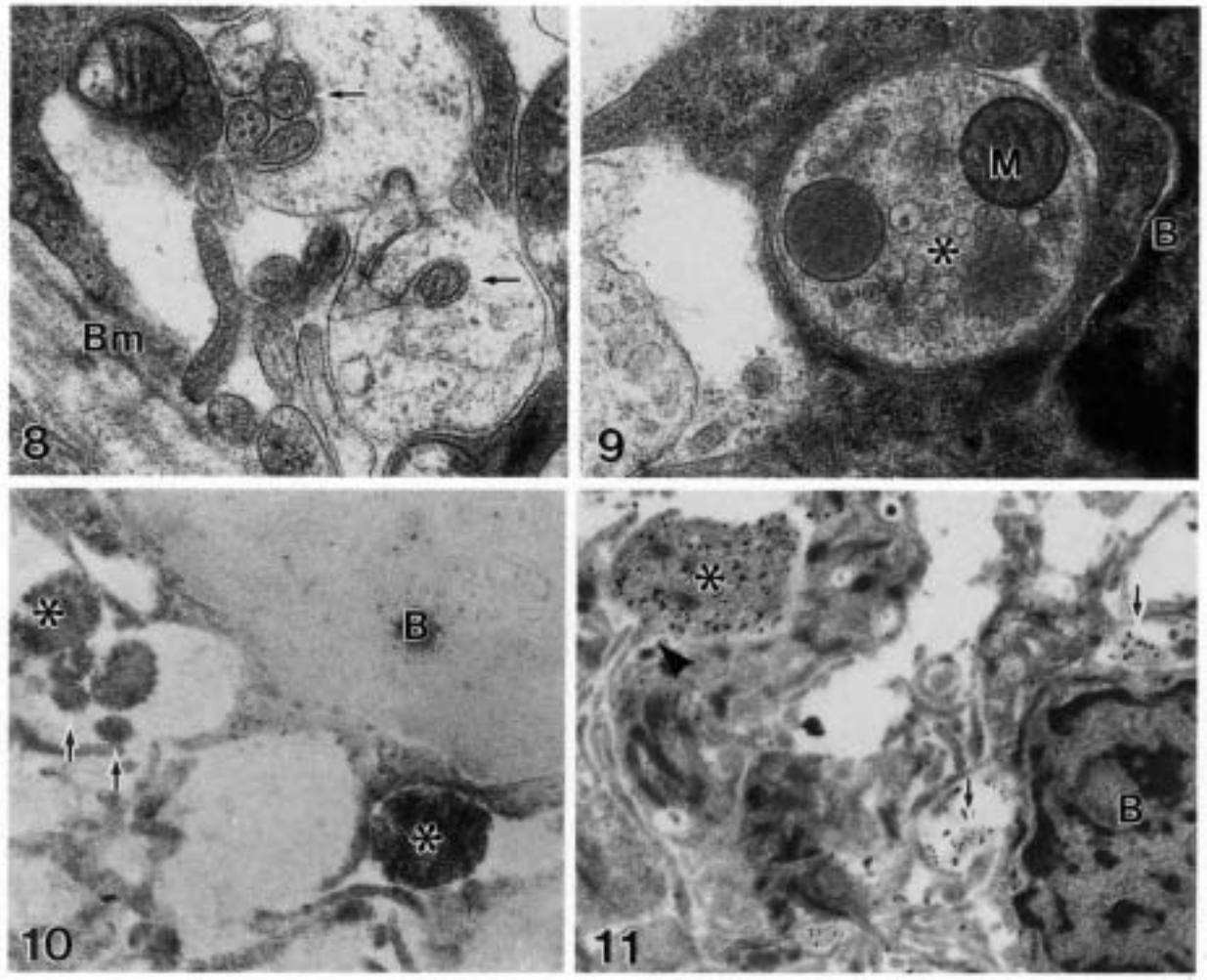
Morphological studies on olfactory receptor cells have been done mainly by means of thin tissue sections. Therefore, it has been difficult to visualize their distribution throughout the nasal mucosa and entire shape of the receptor cells. Whole-mount preparations have been frequently utilized for studies of innervation in the intestine, tracheal mucosa, urethra and other membranous tissues. In the present study, we applied immunohistochemical staining using PGP 9.5 and calbindin antisera to whole-mount preparations of the rat nasal mucosa. As a result, it was possible to gain an overview of the distribution and density of receptor cells and to observe their entire shape, including the dendrite and axon.

Immunostaining using whole-mount preparations: In our preliminary study in which the conventional immunostaining was applied to whole-mount preparations, we failed to obtain a good staining result. The sites immunolabeled with the PGP 9.5 antiserum were restricted to olfactory vesicles exposed on the surface of the olfactory epithelium. In the present study, pretreatment of tissues by freeze-thawing and passage through a graded series of ethanol and xylene greatly improved the staining result, and made it possible to observe the detailed morphology of perikarya and axons, which were located in relatively deeper area of the epithelium. The effect of the pretreatment might have been due to loosening cell junctions by freeze-thawing of tissues and removal of fatty components in the tissue or cell membrane by ethanol and xylene.

Morphology of olfactory receptor cells: The detailed distribution of olfactory receptor cells throughout the nasal septum, including the extension of olfactory area, invasion of receptor cell into the respiratory area, and location of septal organ of Masera, was shown for the first time by the present study. The present study also demonstrated difference in the shape of dendrites extending from perikarya located at various heights of the epithelium. The following reasons are possible explanations for this phenomenon: 1) Olfactory receptor cells are known to be continuously renewed [8, 29]. Since newly differentiated cells have to extend their dendrite to the surface of epithelium through a densely packed cell mass, the extension of dendrite may be mechanically affected, resulting in thin and winding dendrites. 2) Olfactory cells may be classified into subtypes possessing dendrites with different shapes. In either case, we need to do further research about what kinds of factors decide the morphology of dendrites.

Observation of whole-mount preparations, in which basal part of the epithelium was exposed, revealed the detailed morphology of axons. It is generally believed that the axons of receptor cells never form bundles until they penetrate the basal membrane [2]. In the present study, however, the axons were found to gather within the epithelium to form nerve fascicles. In the nasal mucosa of rats, the olfactory area contains as many as 5×10^8 receptor cells [17]. All axons derived from receptor cells project into less than 10^3 glomeruli in the olfactory bulb, where each axon makes synapses with only one glomerulus [16, 27]. Olfactory cells expressing receptors for an identical odor are believed to project to the same glomerulus, but the olfactory cells expressing the same receptors are arranged randomly within the olfactory epithelium [14, 15]. The proximal axons showing a dendric pattern within the epithelium may be reflective of the units of olfactory cells which detect the same odor.

It has been thought that axons extending from olfactory



Figs. 8 and 9. Electron micrographs showing the basal portions of olfactory epithelium. Major population of axons (arrows in Fig. 8) are very thin and contain a few microtubules. A large, membrane-bound nerve element (asterisk in Fig. 9) is observed at the basal region of the epithelium, being held by a basal cell (B). The globular structure contains a few mitochondria (M) and synaptic vesicle-like vesicles. Bm: basal membrane. × 35,000.

Fig. 10. Electron microscopic observation of tissues immunostained for PGP 9.5. Large globular structures (asterisks) as well as thin axons (arrows) are immunolabeled. B: basal cell × 19,000.

Fig. 11. An electron micrograph of a preparation immunolabeled with gold colloidal particles. Gold particles indicating positive immunoreactivity are deposited on both thin structures (arrows) and a globular structure (asterisk). Both structures are connected directly at the position indicated by an arrowhead. B: basal cell × 11,000.

cells are smooth in outline [2], but the proximal axons were found in the present observations to form beaded structures. Since large globular structures with PGP 9.5 immunoreactivity contained synaptic vesicles and were connected with thin axons, this structure was identifiable as a swollen part of axons, like pituitary Herring bodies or varicosity fibers in the autonomic nerve system. The beaded structures may be caused by vigorous axonal flow, which convey synaptic vesicles and other cell organella.

Markers for olfactory receptor cells: Immunohistochemical surveys have shown the presence of several neuron-specific proteins in olfactory receptor cells. In early immunohistochemical studies, NSE and NFP were mainly used as markers of the cells, although antisera against these proteins did not yield constant and intense staining results [20]. In contrast, PGP 9.5 and calbindin are intense in antigenicity and diffusely distributed throughout the cytoplasm of cells. Advantage of PGP 9.5 and calbindin as a marker of olfactory receptor cells was also shown in the present immunostaining. However, the calbindin antiserum did not stain all receptor cells.

The existence of calbindin immunoreactivity in olfactory receptor cells was originally reported in the guinea pig by Iwanaga *et al*., although there were very few calbindin-positive receptor cells [10]. Yamagishi *et al*. [31] proposed that the calbindin-immunoreactive cells in the olfactory epithelium of guinea pigs were the fourth cell type, according to their distributional and morphological features.

Both Fujiwara *et al*. [6] and Johnson *et al*. [12], on the other hand, regarded the calbindin-positive cells, which declined in number with aging, as neuro-secretory cells in mice and humans. In the rat olfactory mucosa, the calbindin-immunoreactive cells were much more numerous than those in the guinea pig and are identified as typical receptor cells as compared with PGP 9.5-immunoreactive cells. This finding suggests that the calbindin antiserum can detect a part of receptor cell population, possibly a subtype of olfactory receptor cells. This idea is supported by data from immunostaining of olfactory nerves by use of antisera against calbindin and other Ca^{2+}-binding proteins. Bastianelli and Pochet [1] reported that calbindin and calmodulin showed different distribution in nerve bundles in the lamina propria of the rat nasal mucosa. There is a possibility that receptor cells as well as olfactory nerves can be classified according to types of Ca^{2+}-binding proteins.

The present study showed that whole-mount preparation of the nasal mucosa for immunohistochemistry is a useful tool to analyze the morphology of olfactory receptor cells. It is expected that this method contributes to studies on ontogeny and degenerative/regenerative processes of olfactory receptor cells.

REFERENCES

1. Bastianelli, E. and Pochet, R. 1994. Distribution of calmodulin, calbindin-D28k and calretinin among rat olfactory nerve bundles. *Neurosci. Lett*. 169: 223–226.
2. Berry, M. M., Standring, S. M. and Bannister, L. H. 1995. Nervous system. pp. 901–1398. *In*: Gray's Anatomy, 38th ed. (Bannister, L. H. ed), Churchill Livingstone, London.
3. Chan, J., Aoki, C. and Pickel, V. M. 1990. Optimization of differential immunogold-silver and peroxidase labeling with maintenance of ultrastructure in brain sections before plastic embedding. *J. Neurosci. Methods* 33: 113–127.
4. Costa, M., Buffa, R., Furness, J. B. and Solcia, E. 1980. Immunohistochemistry localization of polypeptides in peripheral autonomic nerves using whole mount preparations. *Histochemistry* 65: 157–165.
5. Cuschieri, A. and Bannister, L. H. 1975. The development of the olfactory mucosa in the mouse: electron microscopy. *J. Anat*. 119: 471–498.
6. Fujiwara, M., Nakamura, H., Kawasaki, M., Nakano, Y. and Kuwano, R. 1997. Expressions of a calcium-binding protein (spot35/calbindin-D28k) in mouse olfactory cells: possible relationship to neuronal differentiation. *Eur. Arch. Otorhinolaryngol*. 254: 105–109.
7. Graziadei, P. P. C. and Monti, G. G. A. 1979. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspect of differentiation and structural organization of the olfactory sensory neurons. *J. Neurocytol*. 8: 1–18.
8. Hinds, J. W. and McNelly, N. A. 1981. Aging in the rat olfactory system: Correlation of changing in the olfactory epithelium and olfactory bulb. *J. Comp. Neurol*. 203: 441–453.
9. Iwanaga, T., Takahashi-Iwanaga, H., Fujita, T., Yamakuni, T. and Takahashi, Y. 1985. Immunohistochemical demonstration of a cerebellar protein (spot 35 protein) in some sensory cells of guinea pigs. *Biomed. Res*. 6: 329–334.
10. Iwanaga, T., Takahashi, Y. and Fujita, T. 1989. Immunohistochemistry of neuron-specific and glia-specific proteins. *Arch. Histol. Cytol*. 52: 13–24.
11. Iwanaga, T., Han, H., Kanazawa, H. and Fujita, T. 1992. Immunohistochemical localization of protein gene product 9.5 (PGP9.5) in sensory paraneurons of the rat. *Biomed. Res*. 13: 225–230.
12. Johnson, E. W., Eller, P. M. and Jafek, B. W. 1997. Protein gene product 9.5-like and calbindin-like immunoreactivity in the nasal respiratory mucosa of perinatal humans. *Anat. Rec*. 247: 38–45.
13. Johnson, E. W., Eller, P. M. and Jafek, B. W. 1995. Distribution of OMP-, PGP9.5-, and CaBP-like immunoreactive chemoreceptor neurons in the developing human olfactory epithelium. *Anat. Embryol*. 191: 311–317.
14. Kauer, J. S. 1991. Contributions of topography and parallel processing to odor coding in the vertebrate olfactory pathway. *Trends Neurosci*. 14: 79–85.
15. Kauer, J. S. and Moulton, D. G. 1974. Responses of olfactory bulb neurons to odour stimulation of small nasal areas in the salamander. *J. Physiol. (Lond.)* 243: 717–737.
16. Key, B. and Akeson, R. A. 1993. Distinct subsets of sensory olfactory neurons in mouse: Possible role in the formation of the mosaic olfactory projection. *J. Comp. Neurol*. 335: 355–368.
17. Ngai, J., Chess, A., Dowling, M. M., Necles, N., Macagno, E. R. and Axel, R. 1993. Coding of olfactory information: Topography of odorant receptor expression in the catfish olfactory epithelium. *Cell* 72: 667–680.
18. Loo, S. K. 1977. Fine structure of the olfactory epithelium in some primates. *J. Anat*. 123-1: 135–145.
19. Royet, J. P., Souchier, C., Jourdan, F. and Polye, H. J. 1988. Morphometric study of the glomerular population in the mouse olfactory bulb: numerical density size and size distribution along rostrocaudal axis. *J. Comp. Neurol*. 270: 559–568.
20. Takahashi, S., Iwanaga, T., Takahashi, Y., Nakano, Y. and Fujita, T. 1984. Neuron-specific enolase, neurofilament protein and S-100 protein in the olfactory mucosa of fetuses. An immunohistochemical study. *Cell Tissue Res*. 238: 231–234.
21. Tamaki, M., Iwanaga, T., Takeda, M., Adachi, I., Sato, S. and Fujita, T. 1992. Calcitonin gene-related peptide (CGRP)-immunoreactive nerve terminals in the whole mount preparations of the dog urethra. *Arch. Histol. Cytol*. 55: 1–11.
22. Tanaka, J., Ichikawa, R., Watanabe, M., Tanaka, K. and Inoue, Y. 1997. Extra-junctional localization of glutamate transporter EAAT4 at excitatory Purkinje cell synapses. *Neuroreport* 8: 2461–2464
23. Taniguchi, K., Arai, T. and Ogawa, K. 1993. Fine structure of the septal olfactory organ of Masera and its associated gland in the golden hamster. *J. Vet. Med. Sci*. 55: 107–116.
24. Terada, M., Iwanaga, T., Takahashi-Iwanaga, H., Adachi, I., Arakawa, M. and Fujita, T. 1992. Calcitonin gene-related peptide (CGRP)-immunoreactive nerves in the tracheal epithelium of rats: An immunohistochemical study by means of whole mount preparations. *Arch. Histol. Cytol*. 55: 219–233.
25. Thompson, R. J., Doran, J. F., Jackson, P., Dhillon, A. P. and Rode, J. 1983. PGP9.5—a new maker for vertebrate neurons and neuroendocrine cells. *Brain Res*. 278: 224–228.
26. Tos, M. and Mogensen, C. 1977. Density of mucous glands in the normal adult nasal turbinates. *Arch. Oto-Rhino-Larynx* 215: 101–111.
27. Vasser, R., Ngai, J. and Axel, R. 1993. Spatial segration of

odorant receptor expression in the mammalian olfactory epithelium. *Cell* 74: 309–318.
28. Vasser, R., Chao, S. K., Sitcheran, R., Nunez, J. M., Vosshall, L. B. and Axel, R. 1991. Topographic organization of sensory projections to the olfactory bulb. *Cell* 79: 981–991.
29. Weiler, E. and Farbman, A.I. 1997. Proliferation in the rat olfactory epithelium: Age-dependent changes. *J. Neurosci.* 17: 3610–3622.
30. Yamakuni, T., Usui, H., Iwanaga, T., Kondo, H., Odani, S. and Takahashi, Y. 1984. Isolation and immunohistochemical localization of cerebellar protein. *Neurosci. Lett.* 238: 231–234.
31. Yamagishi, M., Nakamura, H., Takahashi, S., Nakano, Y. and Iwanaga, T. 1989. Olfactory receptor cells: Immunohistochemistry for nervous system-specific proteins and re-evaluation of their precursor cells. *Arch. Histol. Cytol.* 52: 375–381.
32. Yoshida, Y. and Takahashi, Y. 1980. Compositional changes in soluble proteins of cerebral mantle, cerebellum and brain stem of rat brain during development: a two-dimensional gel electrophoretic analysis. *Neurochem. Res.* 5: 81–96.