Immunohistochemical Demonstration of Nerve Terminals in the Whole Hard Palate of Rats by Use of an Antiserum against Protein Gene Product 9.5 (PGP 9.5)*

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Summary. Sensory innervation of the entire hard palate was investigated in the rat using serial sections immunostained for protein gene product 9.5 (PGP 9.5), a neuronal marker. PGP 9.5-immunoreactive nerve endings were widely distributed in the hard palate, but the innervation pattern and density differed among portions. They were numerous at papillary protrusions including the incisal papilla, antemolar/intermolar rugae, and postrugal filiform papillae. Immunoreactive free nerve endings gathered at the summits of the connective tissue papillae, some of them entering deeply into the epithelium. Electron microscopy demonstrated that nerves in the postrugal filiform papillae reached the stratum corneum. The atrial region, possibly the most sensitive in the hard palate, showed unique innervation: its anterior part, adjacent to incisors, developed intraepithelial networks of fine and beaded nerves, whereas its posterior part revealed cone-shaped nerve terminals formed on the connective tissue papillae of the atrial folds which comprised two lines of longitudinal flaps. Taste bud-like corpuscles gathered in the medial walls of the incisal canals and in the "Geschmacks-streifen" (taste stripes) present at the most anterior part of the soft palate. The hard palate of the rat is thus richly innervated, and is characterized by region-specific nerve endings which may be involved in mechano- and chemoreception in the oral cavity.

The oral mucosa is morphologically and functionally divided into three types: masticatory, lining, and specialized (TEN CATE, 1998). The mucosa of the hard palate, a thick keratinized tissue, belongs to the masticatory type. This mucosa has been demonstrated to receive a rich supply of nerve fibers and taste bud-like structures—the latter termed chemosensory corpuscles by some researchers—and to function as an integrated sensory apparatus during mastication. Physiological studies have indicated a high sensitivity on the part of the hard palatal mucosa to tactile and pressure stimuli. Clinically, it is well known that denture wearers complain of decreased masticatory efficiency, partially due to a loss of sensory cues from food (CHAMBERS, 1937; MONES, 1950; MURPHY, 1971; BOUCHER et al., 1975; McHENRY, 1992).

Earlier studies using silver impregnation methods revealed a dense innervation in the mucosa of the hard palate in several mammalian species, including some rodents (GAIRNS, 1955; DIXON, 1961, 1962; VIJ and KANAGASUNTHERAN, 1970; SETO, 1972; MUNGER, 1975). Tracer studies by anterograde transport of horseradish peroxidase (HRP)-conjugates injected into the trigeminal ganglion were successful in visualizing nerve terminals in the rat hard palate (CHAN and BYERS, 1985a, b; ARVIDSSON et al., 1995). Although the latter methods demonstrated a more detailed distribution of the trigeminal nerve endings, they failed to visualize the total innervation. Using protein gene product 9.5 (PGP 9.5), a neuron-specific protein, HILLIGES et al. (1996) examined the distribution of the immunoreactive nerves in the human oral mucosa, but their description on the hard palate was fragmental at best. FANTINI et al. (1995) investigated nerve terminals immunoreactive for PGP 9.5 in human oral mucosa, but the observed areas were considerably limited as they used biopsy samples. The same authors noted that the densest and most complete staining of neural structures was obtained
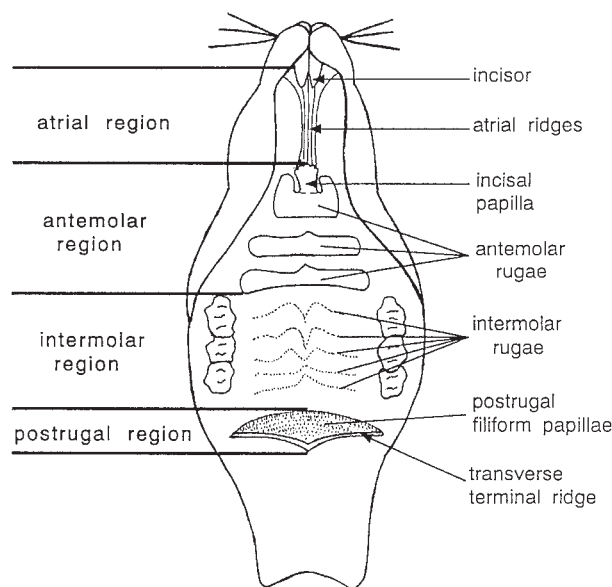
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Fig. 1. A schematic diagram of the hard palate of the rat. It is divided into four regions; each region contains characteristic papillary protrusions listed on right hand of the diagram. An incisal papilla fuses anteriorly with the atrial ridges, and posteriorly with the first antemolar ruga. The postrugal region contains many filiform papillae which are designated as postrugal filiform papillae in this study.

with the PGP 9.5 antiserum, as compared with a panel of antisera against neuropeptides and nerve growth factor (NGF) receptor (FANTINI et al., 1995). 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 the most reliable marker for neurons (THOMPSON et al., 1983). Most previous studies on the innervation of the hard palate in rodents have employed transmission electron microscope (TEM) to focus on areas posterior to the incisal papilla and on the terminal morphology. To our knowledge, no studies are available concerning the innervation of the entire hard palate in any mammal by use of the total neuronal markers. In the present study, serial sections of the rat hard palate cut along different planes were immunostained with a PGP 9.5 antiserum to demonstrate the innervation of the entire hard palate.

MATERIALS AND METHODS

Immunohistochemistry

Adult Wistar rats, weighing 200–300 g, were used in this study. The animals were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and perfused through the ascending aorta with a physiological saline and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The hard palate mucosa and bordering soft palate mucosa were carefully detached from the maxillae and immersed in the same fixative for an additional 6 h. After dipping in 30% sucrose solution overnight at 4°C, tissues were embedded in Tissue-Tek O. C. T. compound (Sakura Finetechnical, Japan), and quickly frozen in liquid nitrogen. Frozen sections, 16 μm in thickness, were serially cut along three different planes (sagittal, frontal and horizontal) and stained by use of the avidin-biotin complex (ABC) method. They were pretreated with 0.3% Triton X-100-containing phosphate buffered saline (PBS) for the enhanced penetration of antibodies, and with 0.03% H_2O_2 in methanol for blocking of the endogenous peroxidase activities. After preincubation with a normal goat serum, the sections were incubated in a rabbit anti-human PGP 9.5 serum (1 : 16,000; Ultraclone, UK). The antigen-antibody reaction was visualized by two consecutive incubations of biotinylated anti-rabbit IgG and ABC (Vector Labs., USA). An enzyme reaction was developed with a mixture of diaminobenzidine (DAB; 0.01%) and H_2O_2 (0.001%) in 0.05 M Tris-HCl buffer, pH 7.6. In some cases, DAB reactions were enhanced by adding 0.04% nickel ammonium sulfate.

Perfusion-fixed maxillae were removed as a whole and immersed en bloc in the same fixative for 6 h. Following fixation, they were decalcified with Plank-Rychlo's solution for 3 days at 4°C. Frozen sections were prepared as mentioned above and stained with hematoxylin-eosin.

Immunoelectron microscopy

Frozen sections, 20 μm in thickness, were prepared from the paraformaldehyde-fixed palatal mucosa, and subjected to the ABC method using the PGP 9.5 antiserum (1 : 16,000 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, briefly stained with lead citrate, and examined with a transmission electron microscope
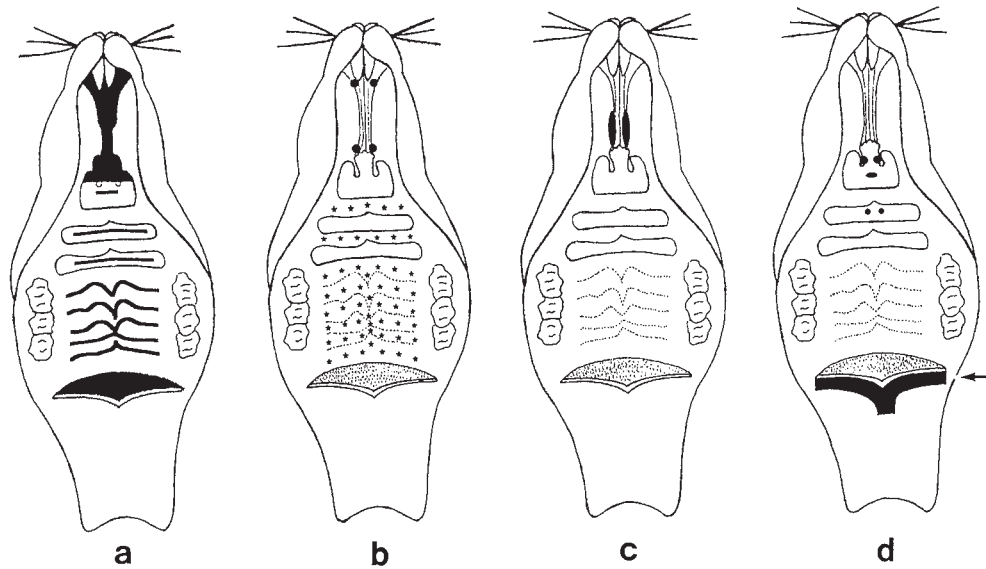
Fig. 2. Schematic diagrams showing the distribution of three types of nerve endings and chemosensory corpuscles, indicated as dark areas, in the rat hard palate. **a.** Intraepithelial free nerve endings are numerous in papillary protrusions and the atrial region. **b.** Bouquet-like and glomerular intraepithelial nerve endings are seen in limited areas of the atrial region. Dendritic nerve endings (*asterisks*), small in size, are dispersed in the valley regions between antemolar/intemolar rugae. **c.** Cone-shaped subepithelial nerve endings gather in the anterior part of incisal papilla. **d.** Chemosensory corpuscles. An *arrow* shows "Geshmacksstreifen" in the soft palate, lined along the boundary between the hard and soft palates.

(TEM) (JEM 100-SX, JEOL, Japan).

RESULTS

For description of the innervation patterns, we subdivided the hard palate into four distinct areas by modifying a classification by KUTUZOV and SICHER (1952) (Fig. 1): 1) The atrial region between the incisors and the incisal papilla. 2) The antemolar region between the incisal papilla and the molars. This region possesses incisal papilla and three antemolar rugae as prominent mucosal protrusions. 3) The intermolar region extending between the molars of both sides and slightly posterior to the last molars. This region contains five M-shaped low rugae, designated as intermolar rugae. 4) The postrugal region, which is a narrow area between the last intermolar ruga and the soft palate. This region is covered with clustered filiform papillae (postrugal filiform papillae) and possesses a transverse terminal ridge at the boundary with the soft palate.

The following three types of nerve endings and taste bud-like structures could be identified, and their

distribution is summarized in Figure 2.

Intraepithelial free nerve endings

Intraepithelial free nerve endings immunoreactive for PGP 9.5 were found essentially throughout the hard palate, but were concentrated at the top of papillary protrusions, such as: 1) incisal papilla, 2) antemolar rugae, 3) intermolar rugae, 4) postrugal filiform papillae, and 5) atrial ridges (see Fig. 2a). In the incisal papilla and three antemolar rugae, numerous immunopositive nerve fibers terminated at the top of connective tissue papillae, and only a small number of them entered the epithelium farther (Fig. 3). In the intermolar rugae and postrugal filiform papillae, the intraepithelial nerve fibers were more numerous and penetrated deeply into the epithelium (Fig. 4), frequently up to the stratum corneum (Fig. 5). Electron microscopic observation confirmed the existence of immunoreactive nerves in the stratum corneum, occasionally very close to the oral cavity (Fig. 6a, b). In the summit of atrial ridges, some single nerve fibers penetrated into the epithelium, but their density was much lower than those in intermolar rugae and postrugal filiform papillae showing a

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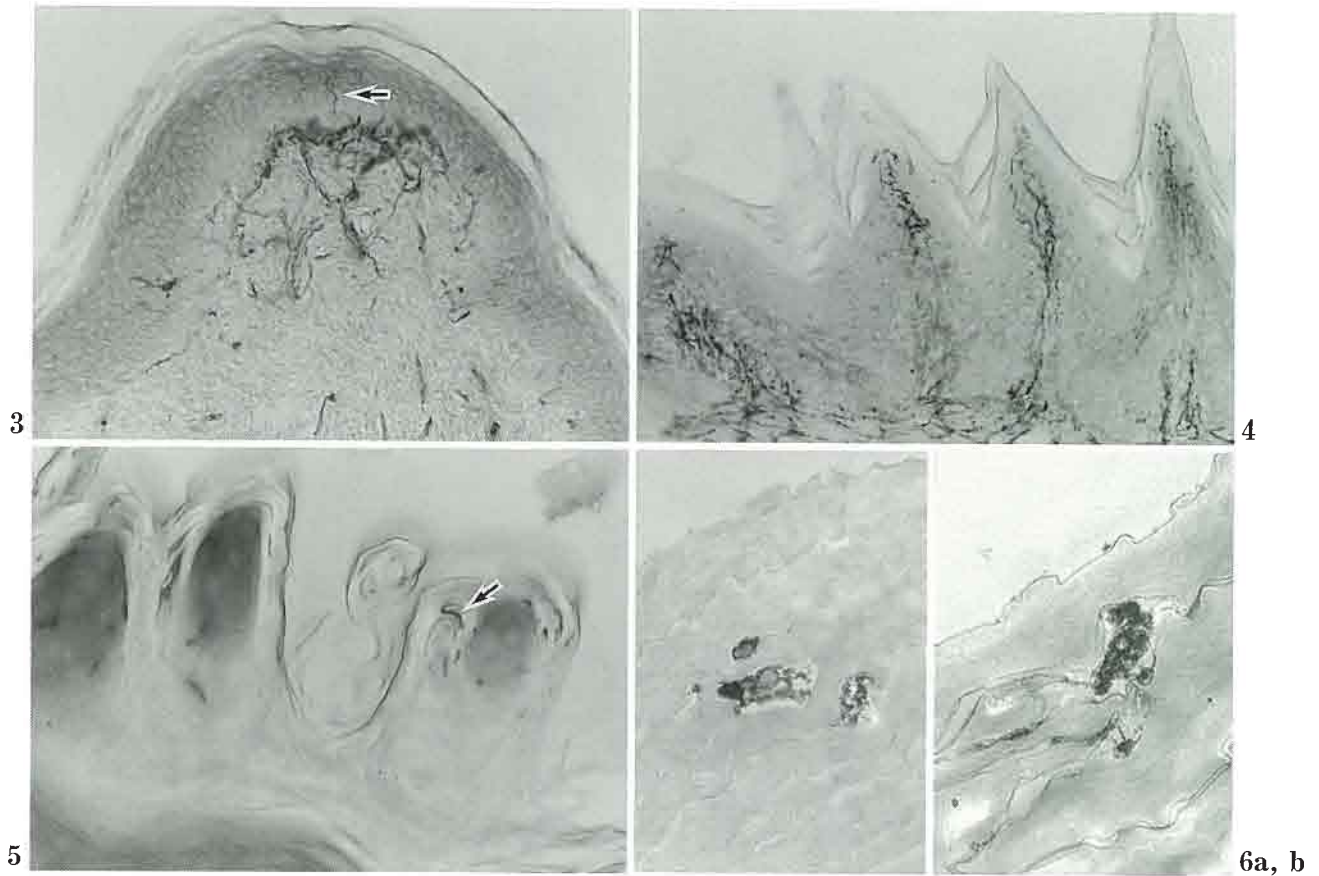
Figs. 3–6. PGP 9.5-immunoreactive free nerve endings in the protruding structures of the rat hard palate. **Fig. 3** shows the innervation of immunoreactive fibers in the second antemolar ruga. Many nerve fibers terminate at the top of connective tissue papillae, some of them further entering the epithelium (*arrow*). In the postrugal filiform papillae (sagital sections, **Fig. 4**), many thin and beaded nerve fibers penetrate deep into the epithelium of each papilla. At a higher magnification of a frontal section cut through the tips of postrugal filiform papillae (**Fig. 5**), PGP 9.5-immunoreactive nerve endings are seen in the stratum corneum (*arrow*). **Fig. 6 a** and **b** are electron micrographs showing PGP 9.5-immunoreactive nerve endings in the stratum corneum of postrugal filiform papillae. Immunolabeled nerves penetrate into the stratum corneum, very close to the oral cavity. Fig. 3 and 4: ×180, Fig. 5: ×350, Fig. 6a: ×8,000, 6b: ×16,500

similar innervation pattern. On the other hand, a transverse terminal ridge at the boundary between the hard palate and soft palate (Fig. 1) contained few nerve fibers intraepithelially.

Another dense distribution of the intraepithelial free nerve endings was found extensively on the lateral sides of atrial ridges in the anterior half of the atrial region (Fig. 7) and on the lateral sides of the incisal papilla (Fig. 8) (also see Fig. 2a). They had a beaded appearance and branched to form a coarse network within the epithelium. Their free ends with slight swellings were in close proximity to the superficial cornified layer.

Characteristic intraepithelial nerve endings

Bouquet-like (Fig. 9) and glomerular nerve endings (Fig. 10) were found intraepithelially in two separate portions of the atrial region, namely just behind the incisors and in front of the incisal papilla (Fig. 2b), which corresponded to the anterior and posterior ends of the longitudinal atrial ridges, respectively. Some of these characteristic nerve endings were located superficially close to the stratum corneum, with others appearing in the basal layer of the epidermis. Another type of specialized nerve terminal, dendritic in appearance, was distributed in the epithelium of valley regions between the rugae in both
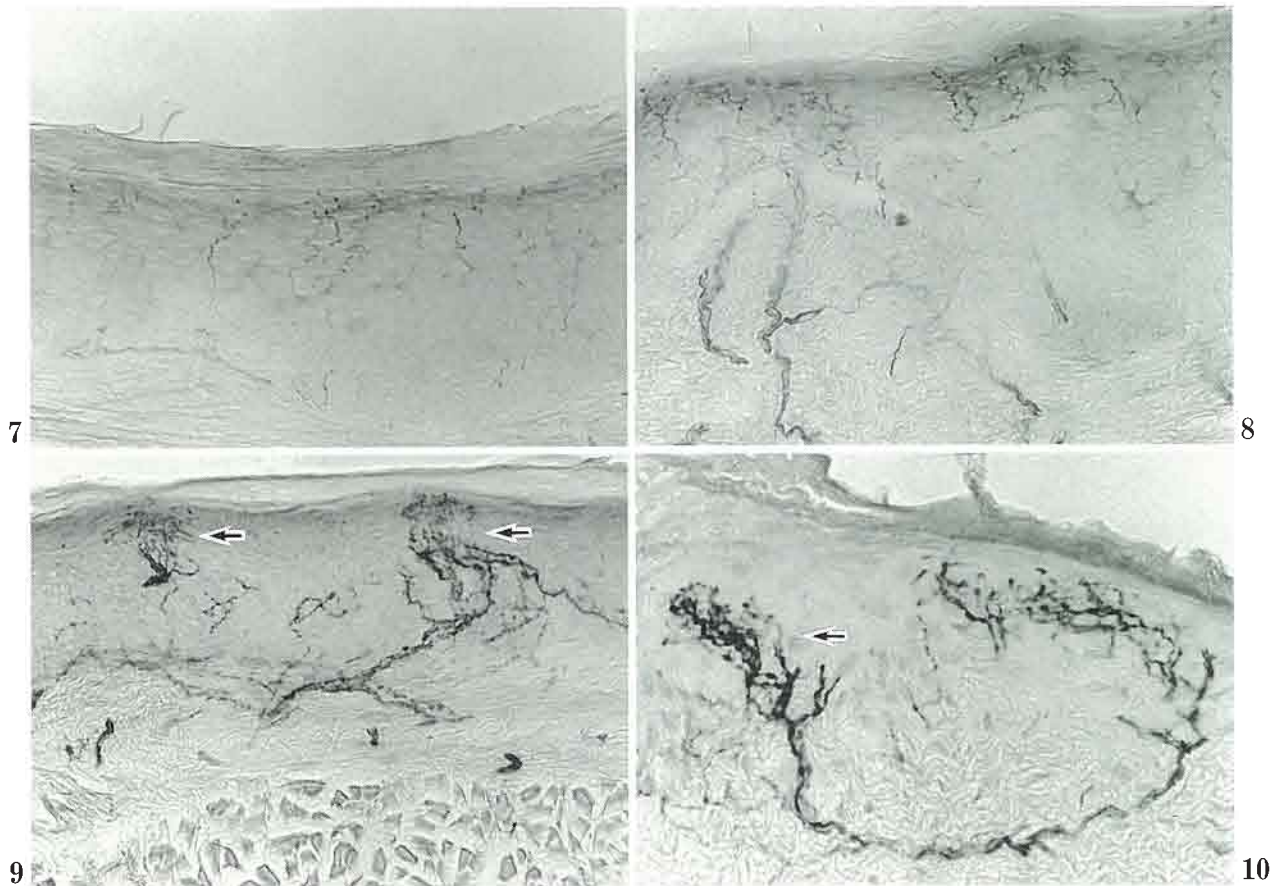
Figs. 7-10. Intraepithelial nerve fibers in the atrial region of the rat hard palate. **Figs. 7** and **8** show intraepithelial free nerve endings in the atrial region with a flat surface, and at the lateral side of the incisal papilla, respectively. Immunoreactive nerves with a beaded appearance form a nerve plexus within the epithelium. In **Figs. 9** and **10**, bouquet-like nerve endings (*arrows* in **Fig. 9**) are located superficially close to the stratum corneum, and a glomerular nerve ending (*arrow* in **Fig. 10**) is found at the basal layer of the epidermis. Figs. 7-9: ×180, Fig. 10: ×350

antemolar and intermolar regions (Fig. 2b, asterisks). This type of nerve ending was fragmented or extended in relatively small areas.

Characteristic subepithelial nerve endings

The atrial region contained special nerve terminals gathering in restricted regions of the subepithelium (Fig. 2c). The atrial region carried about five rows of small ridges (atrial ridges), which ran parallel along the midline of the atrial region from the incisors to the incisal papilla (Fig. 1). The mucous membrane was conspicuously elevated on each lateral side of the atrial ridges to form a tall, sharp longitudinal fold that inclined medially and was sickle-shaped in cross sections (Figs. 11, 12). These folds—named the atrial

folds by KUTUZOV and SICHER (1952)—from both sides appeared to meet each other with their tips and formed an incomplete sheath over the atrial ridges. At their base, they possessed dispersed connective tissue papillae of various height, some of them extending deeply into the atrial fold.

In the posterior part of these atrial folds (Fig. 2c), nerve fibers ran into the connective tissue papillae and showed a unique arrangement. The characteristic nerve terminals, cone-shaped as a whole, were clearly visualized in sagittal sections (Fig. 13). Individual fibers ran along the surface of the connective tissue papillae and terminated at their top (Figs. 12, 14). Some of the nerve fibers further penetrated into the epithelium, but their course was restricted to the basal layer of the epithelium.
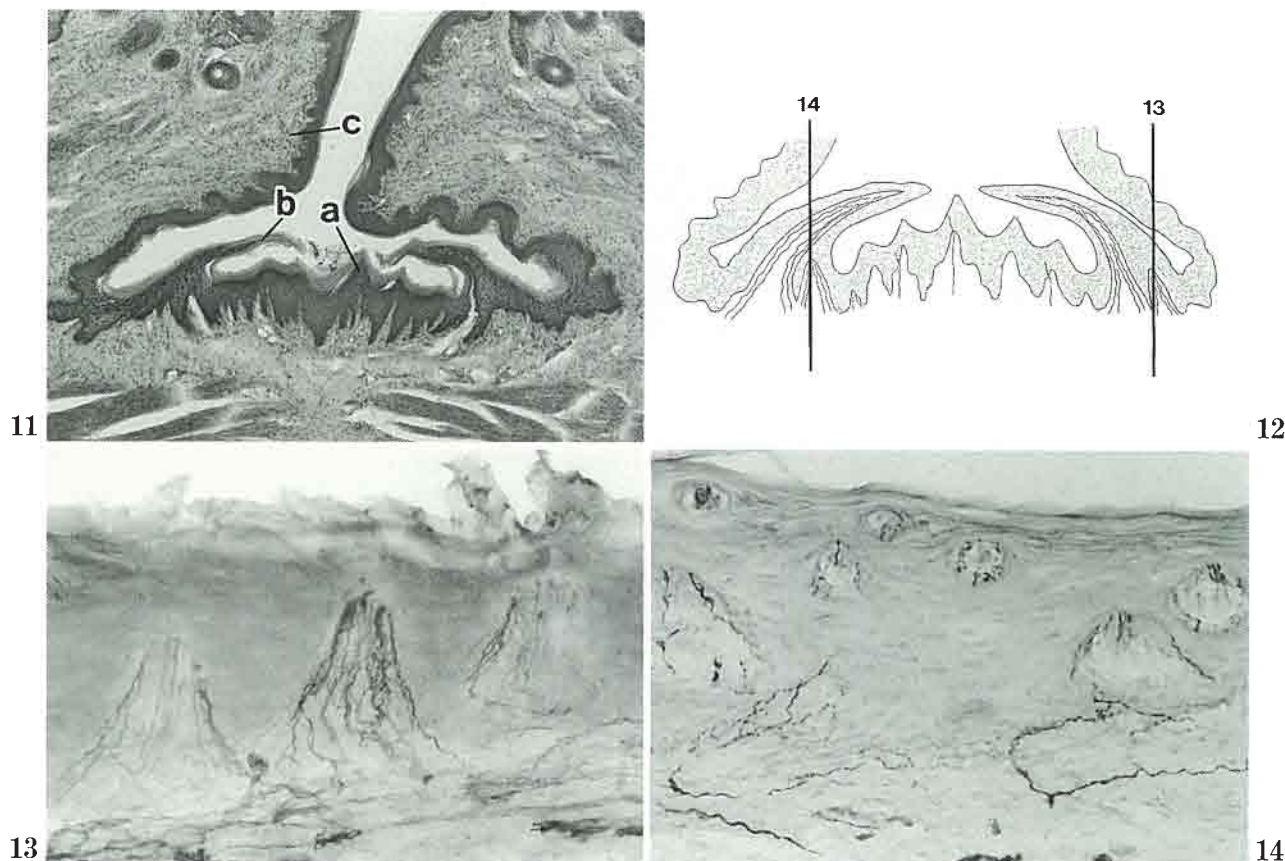
Figs. 11–14. Innervation in the atrial folds and the atrial ridges. **Fig. 11** shows a frontal section through the atrial region (hematoxylin-eosin staining). *a* Atrial ridges, *b* the atrial folds, *c* labial folds. A schematic diagram (**Fig. 12**), corresponding to Figure 11, shows the innervation in the atrial region at a frontal section. Two lines marked by the numbers (*13, 14*) show sagitally cut planes in Figures 13 and 14. Three cone-shaped, subepithelial nerve endings occupy and cover the connective tissue papillae of the atrial folds (**Fig. 13**). Individual fibers run along the surface of the connective tissue papillae. In **Fig. 14**, several connective tissue papillae are cut obliquely. Fig. 11: ×40, Fig. 13 and 14: ×180

Chemosensory corpuscles

Immunostaining for PGP 9.5 clearly demonstrated the localization of taste bud-like structures, called chemosensory corpuscles by previous researchers (CHAN and BYERS, 1985a, b; LIEM et al., 1990a, b: ARVIDSSON et al., 1995) (Fig. 2d). They gathered in the medial walls of the incisal canals (Fig. 15). A small number of the corpuscles were also encountered at the first and second antemolar rugae, although the first ruga was not a prominent peak due to partial fusion with the incisal papilla (cf. Fig. 1). In the soft palate, we confirmed the existence of many chemosensory corpuscles distributed on lines in the most anterior part (Fig. 2d, arrow), which are known as "Geschmacksstreifen" (taste stripes) (KAPLICK, 1953).

Numerous PGP 9.5-immunoreactive nerve fibers were densely distributed in the connective tissue beneath the corpuscles (Fig. 15). A number of nerves penetrated the epithelium adjacent to the corpuscles running toward the surface of the epithelium (perigemmal nerve fibers) (Fig. 16). The immunoreactive fibers also entered the basal side of the corpuscles and terminated inside (intragemmal fibers). The intragemmal nerve fibers were less numerous than the perigemmal ones surrounding the corpuscles. In the corpuscles, not only nerve fibers but also some constituent cells showed immunoreactivity for PGP 9.5, albeit with different intensity. They appeared as slender spindles and possessed an oval nucleus (Fig. 16).
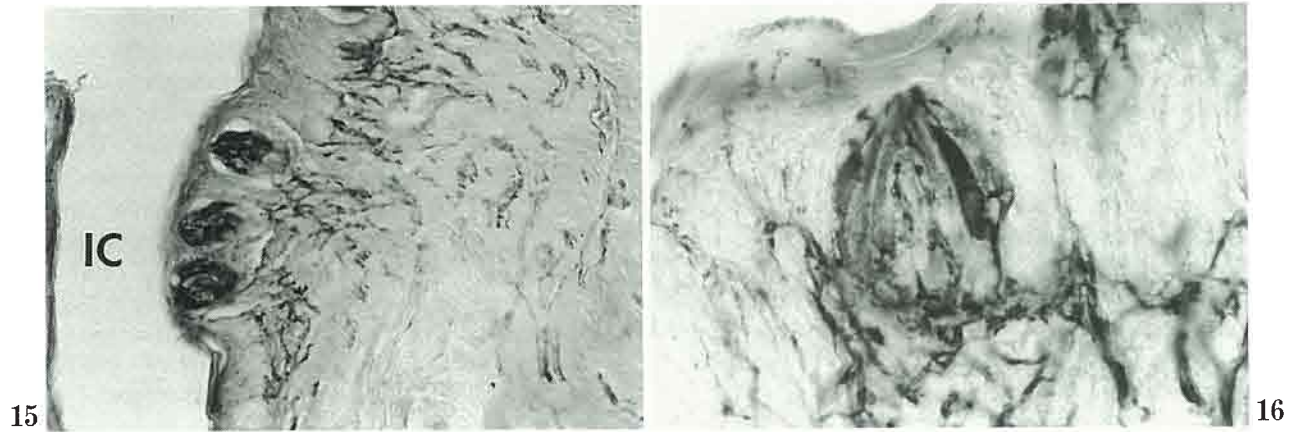
Figs. 15 and 16. Chemosensory corpuscles in the medial wall of the incisive canal (*IC*). High magnification of a corpuscle (**Fig. 16**) shows immunoreactive nerves distributed in the surrounding epithelium as well as within the corpuscle. One cell of the corpuscle is intensely positive, while the other cells react weakly. Fig. 15: ×180, Fig. 16: ×300

DISCUSSION

The hard palate is rich in nervous elements, being comparable to the teeth and periodontium. The present immunohistochemical study by the use of PGP 9.5 antiserum is the first to demonstrate the density and terminal morphology of nerve fibers in the whole hard palate of rats, and the difference in their distribution due to location. Since the nerve fibers in the hard palate are distributed exclusively in the epithelium and subepithelial connective tissue, they may be sensory in nature. Mechanical stimuli to the hard palate are known to induce delicate reflex responses of the oral musculature in the cat (THEXTON, 1973; HELLSTRAND, 1982; TAKATA et al., 1991, 1992; TOMIOKA et al., 1997, 1999), rat (VAN WILLIGEN and WEIJS-BOOT, 1984) and in humans (SMITH et al., 1985). Generally, the protruding portions in the palatal mucosa such as the incisal papilla and antemolar/intermolar rugae contained many free nerve endings, and seemed favorable for mechanoreception. In the protrusions in the antemolar region (incisal papilla and antemolar rugae), most of the nerves terminated at the top of connective tissue papillae, while the intermolar protrusions (intermolar rugae) were characterized by the frequent penetration of nerves into the epithelium. WEIJS-BOOT and VAN WILLIGEN (1978) and VAN WILLIGEN and WEIJS-BOOT (1984) reported that reflex responses of the oral musculature to electrical and mechanical stimulation of the hard palate in the rat differed between the antemolar and intermolar regions. These different responses may be accounted for partially by the difference in the innervation patterns in the protruding structures of the palatal mucosa.

Previous studies have reported the distribution of nerve endings in the hard palate of some rodents by silver impregnation methods (DIXON, 1961, 1963), a tracer labeling method (YEH and BYERS, 1983; BYERS and YEH, 1984; CHAN and BYERS, 1985a, b; LIEM et al., 1990b; ARVIDSSON et al., 1995) and immunohistochemistry (HIRATA et al., 1988; LIEM et al., 1990b; ITOTAGAWA, 1990; MIYAWAKI et al., 1996; ICHIKAWA and SUGIMOTO, 1997; KATO et al., 1998). However, there are no studies which have dealt with innervation in the whole region of the hard palate. ARVIDSSON et al. (1995) used anterograde transport of HRP conjugates to demonstrate Ruffini endings in the incisal papilla, subepithelial endings in protruding parts, and intraepithelial endings with Merkel cells in the rat hard palate. Although the study by ARVIDSSON et al. (1995) covered considerably broad areas of the rat hard palate, it lacked information on the innervation of the atrial region, which appears to be most sensitive, judging from the richness of its innervation. The present study thus has been able to reveal a detailed innervation pattern and specialized nerve terminals, including the cone-shaped terminals in this area described below.

The posterior part of the atrial region develops long folds which are a filmy sheath over the atrial ridges. These atrial folds possess a cone-shaped sensory apparatus showing a unique arrangement of nerves in the connective tissue papillae. Although the functional significances of the characteristic struc-

tures are obscure, their morphological features suggest that they are flexible and easily forced down by food. Thus, mechanoreception by the atrial folds is quite a reasonable concept. The atrial region, which is situated between incisors and incisal papilla, is not present in the human oral cavity, and its functions are unknown. However, we found that this region is also rich in specialized nerve terminals including the bouquet-like and glomerular intraepithelial nerve endings. It is conceivable that nerve endings in this region play an important role in transporting food particles to the posterior part and in determining the size of food particles.

On the other hand, the anterior part of the atrial region developed coarse networks of free nerve endings within the epithelium, especially at the lateral sides of both atrial ridges and incisal papilla. Their terminals tended to extend to the stratum corneum. Another rich existence of intraepithelial nerves was found in the postrugal filiform papillae, some of them apparently reaching into the stratum corneum. The present electron microscopy confirmed the deep invasion of intraepithelial nerves into the stratum corneum, suggesting a possibility that taste stimuli easily reach them. It is worth noting that the human hard palate does not contain any taste buds (Travers and Nicklas, 1990), but some denture wearers complain of taste disorders (Chambers, 1937; Mones, 1950; Boucher et al., 1975). Murakami et al. (1995) reported that wearing an experimental palatal plate dulled the sensation of both sour and bitter. From these experimental and clinical findings, there is a possibility that the free nerve endings entering deep into the epithelium may function as taste receptors. However, further studies are needed in order to clarify how deeply food-derived substances penetrate through the epithelium and what kinds of tastes might be detected by this type of nerve.

The present immunohistochemical study confirmed previous findings on the taste bud-like structures (chemosensory corpuscles) in the incisal papilla and in the most anterior part of the soft palate, the latter called "Geschmacksstreifen" (taste stripes) (Kaplick, 1953). Several studies have described the distribution of these corpuscles in the rat hard palate (Yeh and Byers, 1983; Chan and Byers, 1985a; Settembrini, 1987; Hirata et al., 1988; Arvidsson et al., 1995). The innervation pattern of the corpuscles was almost same as that of taste buds on the lingual surface as shown by previous immunohistochemistry for PGP 9.5 (Iwanaga et al., 1992; Kanazawa and Yoshie, 1996). These authors have identified the PGP 9.5-immunoreactive spindle-shaped cells as Type III cells, possibly gustatory cells, on the basis of their forming

synapses with nerve terminals. In the present study, we also found a small number of the same corpuscles on the antemolar rugae. The corpuscles in this region were reported by Takahashi (1998) to decrease in number or disappear in aged animals. The same author also noted that they might have some function related to suckling, but not be involved in taste reception because of absence of any taste pores in the corpuscles.

The present study showed that the hard palate of the rat is richly innervated and contains some characteristic nerve endings, which are considered mechano- and chemoreceptors important for oral sensation.

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