

Morphological differences among nerve fiber endings in the rat oral mucosa as revealed by methylene blue staining

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Summary. The nerve fiber distribution in the oral mucosa of the soft palate and palatoglossal arch of the rat was studied by means of methylene blue supravital staining. It was focussed primarily on the dye uptake of intraepithelial nerve fibers. Differences in the morphology of nerve fiber terminations were found between these regions of the oral mucosa. In the soft palate, local accumulations of intraepithelial nerve fibers which branched and showed terminal enlargements were detected. Intra- and perigemmal nerve fibers of chemosensory corpuscles could be stained. In the palatoglossal arch, numerous elongated papillae were seen containing nerve fiber plexus showing a complicated arborization pattern. In part, the collaterals penetrated the epithelium. The soft palate contained only a small number of lower but broader papillae which were covered by a more expanded intraepithelial nerve fiber plexus. In both regions, anastomoses between the branches of single nerve fibers were sometimes seen. Solitary delicate nerve fiber endings were loosely distributed throughout the epithelia. In addition, intrapapillar nerve endings, which were enclosed by a slightly stained capsule, were intensely stained; they showed characteristic lateral protuberances. The Merkel's discs were visualized as the components of a terminal network of nerve fiber branches. The observed differences in the shape and locations of the nerve terminations suggest different functions of these nerve fibers. Due to the low costs of the staining procedure and its ease in handling, it is well-suited for a mapping of the innervation pattern of the whole oral mucosa.

Key words: Epithelium, Innervation, Taste buds, Merkel cell, Encapsulated corpuscles

Introduction

Most light microscopic data on the innervation of the oral mucosa are based on early studies performed by means of supravital methylene blue (MB) staining and silver impregnation (Botezat, 1901; Gairns, 1955; Kadanoff and Gürowski, 1963). MB-staining of nerve fibers is achieved by intracardiac, intravascular or submucous dye injection followed by exposing the tissues to the air (Ehrlich, 1886). When the development of the staining process has reached its optimum, fixation is carried out which leads to a precipitation of the dye and a preservation of the tissue structure. Using this technique, Botezat (1901) obtained images showing interesting morphological details regarding the innervation of the connective tissue papillae, free intraepithelial nerve fiber endings and the Merkel cell innervation in the mucosa of the hard palate of different mammals. Unfortunately, all the obtained images were only documented by drawings. Moreover, they could not be reproduced for a long time. This problem was attacked again (Dixon, 1961; Yamamoto et al., 1986), but minute morphological characteristics like the shape of the intraepithelial nerve fiber endings were not investigated in these studies.

The ultrastructure of different nerve endings in the oral mucosa has been investigated by Halata and Munger (1983) and Tachibana et al. (1987).

Meanwhile, a series of neuron-specific proteins and neuropeptides were immunohistochemically detected within nerve fibers innervating the oral mucosa; some were also visualized in the palate (Rodrigo et al., 1985; Itotagawa, 1990; Ramieri et al., 1992). Moreover, the innervation of the hard palate of the rat was investigated using labelling with conjugated horseradish peroxidase (Arvidsson et al., 1995). Nevertheless, regarding the morphological details the resulting images of these studies did not reach in every respect those drawings which had been obtained by MB staining (Botezat, 1901). Due to the fact that the former descriptions regarding the MB staining method were only very brief,

a modification of this procedure was worked out by the author of the present study to allow the production of paraffin sections of stained tissues without loss of dye; no heavy precipitations of the reaction products obscure the plexus of delicate nerve fibers (Müller, 1994). Therefore, this technique is now applied for a closer examination of the innervation pattern of the mucosa of the soft palate and palatoglossal arch of the rat. These regions of the oral mucosa were chosen for the investigation because only few data on their nerve fiber distribution exist in the literature (see Rodrigo et al., 1985). Moreover, it was intended to demonstrate that delicate regional differences in the innervation pattern between these closely neighbored zones of the oral mucosa can be visualized by methylene blue staining.

Materials and methods

Adult Sprague-Dawley rats were used for the experiments. The animals were killed with diethyl ether. Immediately thereafter, a 0.25% dye solution (MB med. puriss., C.I. 52015; Chroma, Köngen, Germany) was injected subepithelially into the soft palate and the palatoglossal arch of the animals. The oral mucosa was then removed and exposed to the air in a moist chamber for 45 minutes at 20 °C.

Fixation and embedding in paraffin were done in a modified manner according to the author's descriptions (Müller, 1994): the first fixation was performed at 4 °C (refrigerator) for 5 hours (stock solution: 100 ml of a 9% aqueous ammonium heptamolybdate solution with the addition of 9 drops of 25% hydrochloric acid and 0.9 ml 30% hydrogen peroxide). After a short rinse in distilled water, a 2nd fixation took place for 2 hours 30 minutes at 4 °C [stock solution: 100 ml of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 1.8% phosphomolybdic acid and 0.1% hydrogen peroxide (final pH 5.0)]. Subsequently, the specimens were washed overnight in distilled water.

The tissues were dehydrated in 100% tertiary butanol (melting point: 25 °C) for 48 h. The first alcohol change was performed after 15 minutes, the second after 1 hour and the third after 7 hours. For these three preliminary dehydration steps, phosphomolybdic acid was added to the alcohol in a concentration of 0.05%. The tissues were then transferred into pure tertiary butanol. After dehydration, they were stored for 1 hour in a mixture of 8 parts decahydronaphthalene (Dekalin®; Chroma, Köngen, Germany) and 2 parts methyl benzoate. Before being embedded in paraffin, they were immersed for another hour in 100% decahydronaphthalene. 20 to 60 µm-thick sections were mounted on glass slides. After drying, they were deparaffinized in xylene and coverslipped with DePeX® (Serva, Heidelberg, Germany). Due to the thickness of the paraffin sections, combined with the 3-dimensional structures of the nerve fiber complexes, it was focussed and photographed in different planes. Photomontages were therefore required to visualize the shape and routes

of the nerve fibers.

Results

In the soft palate as well as in the palatoglossal arch, intensely blue stained nerve fibers were observed within the subepithelial connective tissue; in part delicate branches also penetrated the epithelium (Figs. 1-20). Within the underlying connective tissue, anastomoses between nerve fiber branches appeared to occur (Fig. 2). In the lamina epithelialis, a subpopulation of nerve fibers were also seen in an intimate association with each other (Fig. 3). Most of the nerve fibers showed knob-like terminations within the superficial cell layers (Fig. 4).

In the soft palate, local accumulations of intra-epithelial nerve fibers were observed which ended with characteristic enlargements (Fig. 5); very often, these nerve fiber terminals showed spiny appendages (Fig. 6). In addition, the innervation of chemosensory corpuscles (taste buds) could be visualized in the vicinity, i.e. intragemmal as well as perigemmal nerve fibers were stained. The perigemmal nerve fibers originated from the basal plexus and formed together with the intragemmal nerve fibers an intensely innervated compartment of the epithelium (Figs. 7, 8).

In both parts of the oral mucosa, the connective tissue papillae represented the locations of special nerve fiber plexus (Figs. 9-12). Differences were seen between the soft palate and the palatoglossal arch because the last mentioned area showed a large number of elongated cone-shaped papillae (Fig. 9), whereas the soft palate contained only a smaller number of lower but broader papillae (Fig. 10). These differences in the shape of the papillae also altered the morphology of their nerve fiber complexes (Figs. 9, 10). The arborization of the nerve fiber plexus at the top of the papillae in the palatoglossal arch showed a characteristic shape: in most cases, a single nerve fiber stretched up to the top of the papillae, where it branched; most of the collaterals entered the epithelium. The branching point very often showed a typical swelling (Figs. 11, 12). Intraepithelially, the branches formed terminal enlargements of different morphology: longish and bulbous swellings were seen, in part covered by spine-like protuberances (Figs. 12, 13). Infrequently, lanceolate endings resembling those which are arranged like palisades around the hair follicles in the skin could also be detected (Fig. 14). Regarding the nerve fiber plexus formation at the top of the papillae in the soft palate, the nerve fiber took its route at the base of the epithelium sending off collaterals into the epithelium (Fig. 10). Sometimes, the intra-papillar nerve fiber formed a complete loop representing an anastomosis between the branches of a single nerve fiber (Fig. 15).

In both regions of the oral mucosa, solitary nerve fiber endings were intensely stained within the connective tissue papillae; they showed no branching and were enclosed by a slightly stained capsule. The encapsulated nerve fiber showed spiny protuberances

(Fig. 16).

At the base of the epithelia of both mucosal regions, nerve fiber plexus ending in the shape of laminar expansions, which were identified as Merkel's discs, could be visualized (Figs. 17-20). The Merkel cells themselves remained completely unstained. The closer investigation of Merkel's discs and the nerve fiber branches which were involved in their formation revealed the occurrence of anastomoses in the shape of a terminal network (Figs. 19, 20).

Discussion

The most interesting result of the present study was the observation that MB staining is not only capable of

visualizing terminal nerve fibers in the oral mucosa of the rat. Moreover, the method applied here enables the investigator to study the delicate branches of the nerve fibers more closely than it is possible by immunohistochemistry or labelling with conjugated horseradish peroxidase. This is caused by the phenomenon that the dye is selectively stored by the nerve fibers under supravital conditions followed by a transformation of the dye to a stable fine-grained precipitate during fixation; this reaction product is not dislocalized during the subsequent treatment, especially dehydration (see also Müller, 1990).

In particular, the discussed advantages of the staining procedure revealed the visualization of the routes, ramifications and terminations of the intra-

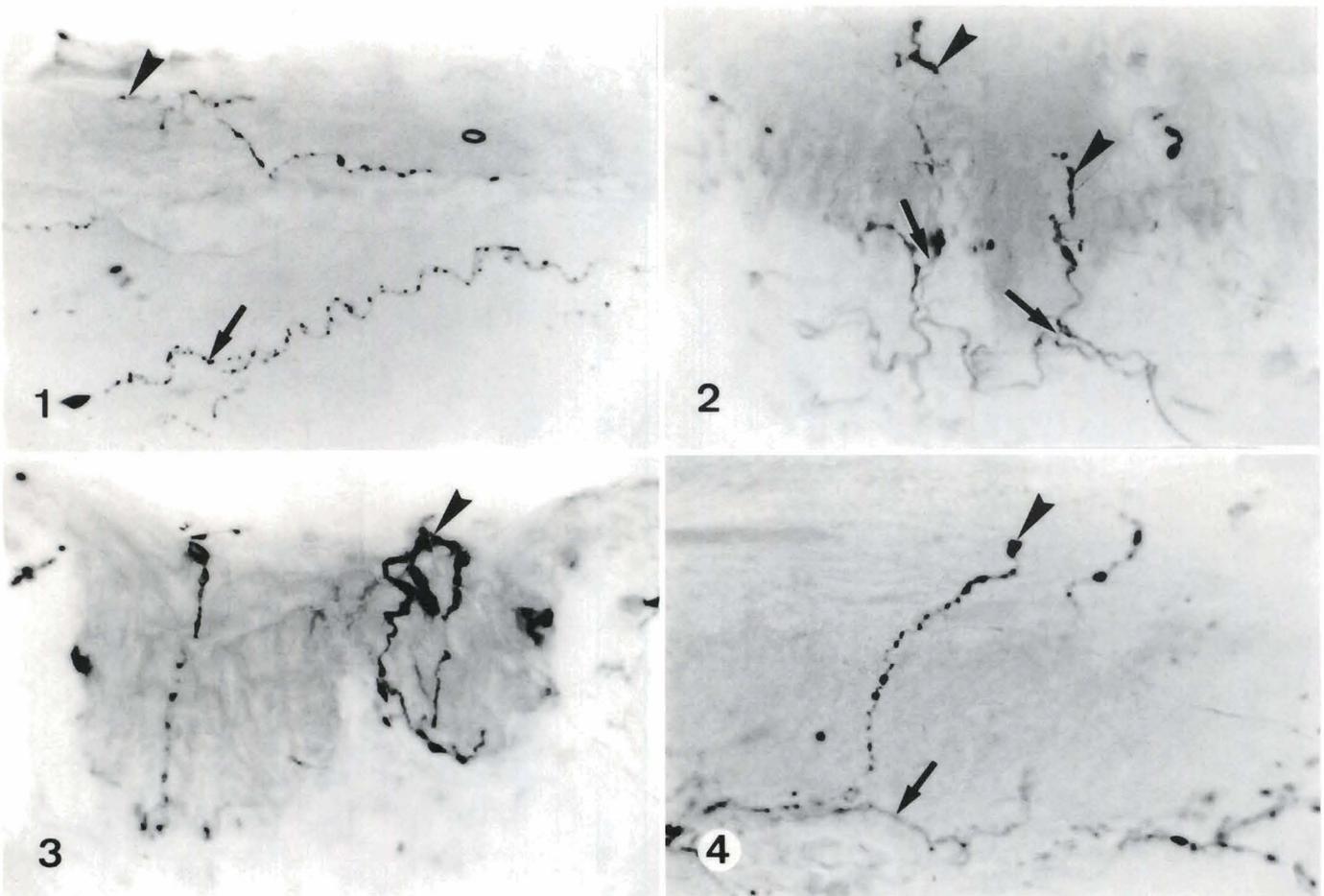


Fig. 1. Soft palate. A nerve fiber (arrow) is visible in the subepithelial connective tissue. Close to the surface of the mucosa, the termination of a thin intraepithelial nerve fiber (arrowhead) after branching is also stained. x 410

Fig. 2. Palatoglossal arch. Nerve fiber branches (arrowheads) stretch up from the subepithelial nerve fibers. Anastomoses (arrows) seem to occur between neighbouring nerve fibers within the subepithelial connective tissue. x 920

Fig. 3. Palatoglossal arch. Two delicate intraepithelial nerve fibers appear to be in contact with each other (arrowhead). x 430

Fig. 4. Palatoglossal arch. The thin intraepithelial nerve fibers show knob-like endings (arrowhead). They represent branches of the subepithelial nerve fibers (arrow). x 850

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epithelial nerve fibers. It could be shown that different morphological types of these nerve fibers occur in the oral mucosa of the soft palate and the palatoglossal arch of the rat. Furthermore, it was demonstrated that topographic differences in the innervation pattern also exist between both mucosal regions. Common to both regions was the most frequently occurring type of delicate intraepithelial nerve fiber branches which left the nerve fibers at the base of the epithelium and stretched up to the superficial cell layers ending with characteristic enlargements.

Interestingly, within the soft palate, accumulations of intraepithelial nerve fibers were found forming intensely innervated epithelial compartments. Such zones of intense innervation have not been described in the literature up to now. They might represent special sensory units in this region of the oral mucosa.

Moreover, it has to be pointed out here that the innervation of the taste buds in the soft palate in the shape of the intra- and perigemmal nerve fibers has to be regarded as an additional sensory property of this region. These nerve fibers are considered to be of chemosensory nature in taste reception, whereas the concrete function of the other intraepithelial nerve fibers remains a matter of speculation and cannot be clarified by a pure morphological investigation. Nevertheless, it has to be pointed out that the delicate ascending intraepithelial nerve fibers far away from taste buds very often resemble the perigemmal ones and might perhaps fulfill similar tasks. Here, it has to be pointed out that the concrete functions of the intraepithelial nerve fibers cannot be clarified by a pure morphological study. Nevertheless, it could be shown electrophysiologically that the two sensory modalities, taste and mechano-

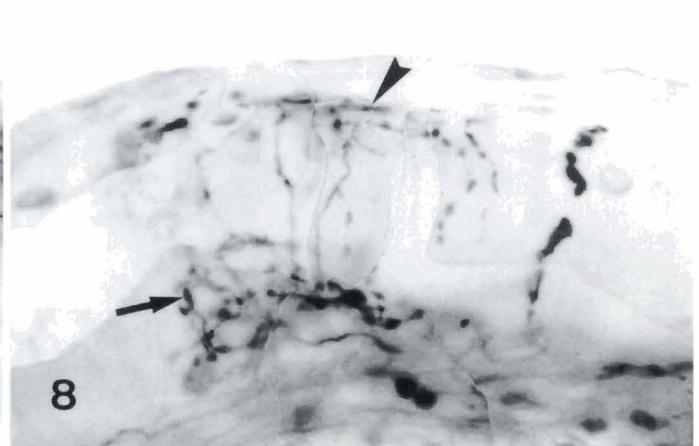
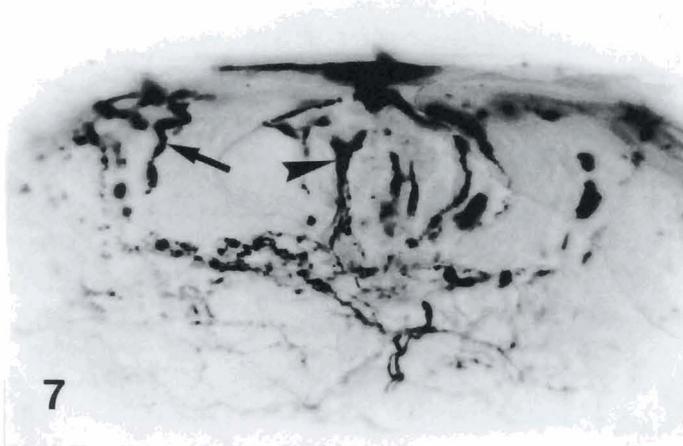
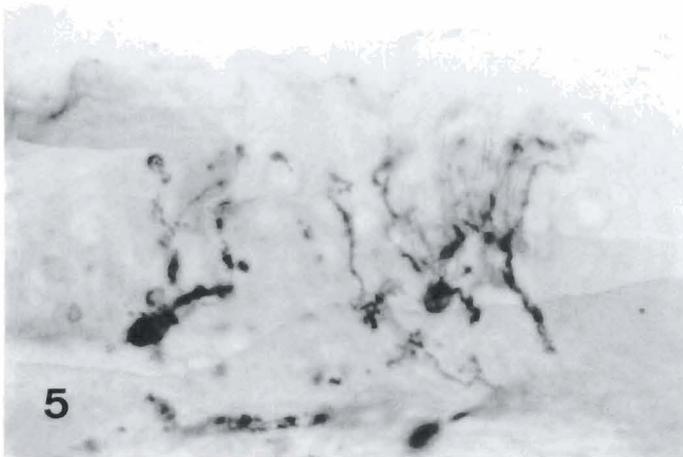


Fig. 5. Soft palate. Intensely innervated epithelial compartment. x 690

Fig. 6. Soft palate. The knob-like endings of the intraepithelial nerve fibers sometimes show spine-like protuberances (arrowhead). x 1,530

Fig. 7. Soft palate. The innervation of chemosensory corpuscles (taste buds) also becomes visible in the shape of intragemmal nerve fibers (arrowhead) and perigemmal nerve fibers (arrow). x 530

Fig. 8. Soft palate. The perigemmal nerve fibers ascend from a dense basal plexus (arrow). Subsequently, they branch and terminate close to the surface (arrowhead). x 768

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reception, from the rat oral mucosa may converge on one neuron (Ogawa et al., 1982). This would also suggest that nerve fibers which reach the superficial cell layers

like the perigemmal ones etc, might be responsible for chemoreception as well as low threshold mechano-reception.

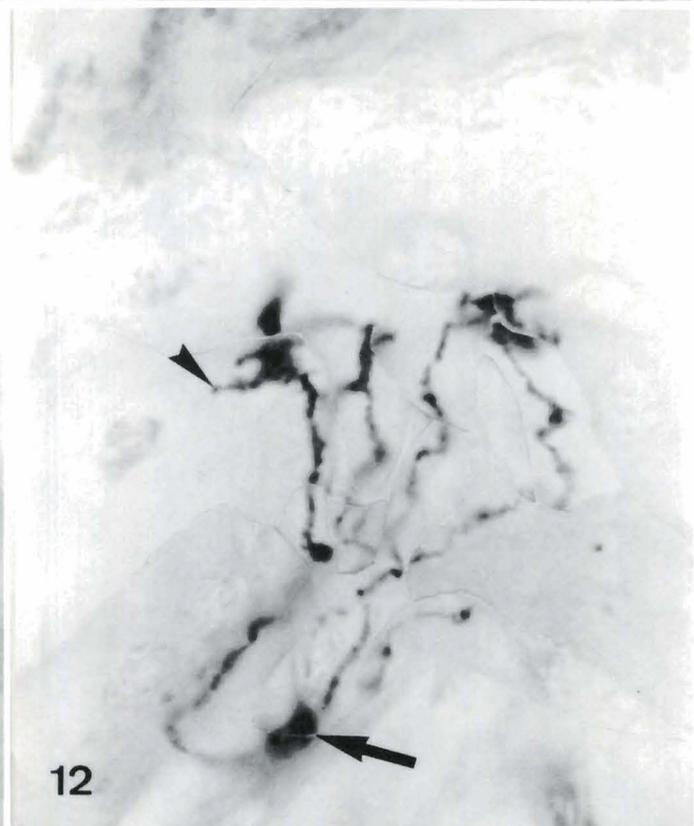
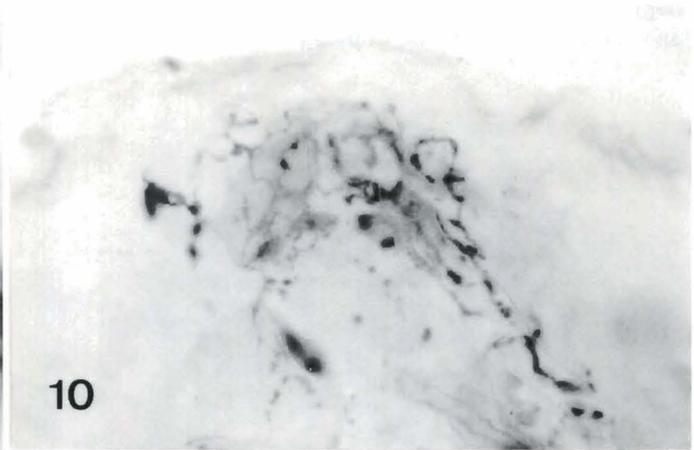


Fig. 9. Palatoglossal arch. On top of the papillae, intensely stained intraepithelial nerve fiber plexus are found. The epithelium itself is only slightly stained. x 630

Fig. 10. Soft palate. In this part of the oral mucosa, the connective tissue papillae are broader; therefore intraepithelial nerve fiber plexus on top of the papillae are more expanded. x 670

Fig. 11. Palatoglossal arch. The nerve fiber branches entering the epithelium at the top of the papillae arise very often from an enlargement (arrow) of the intrapapillar nerve fiber. x 1,000

Fig. 12. Palatoglossal arch. The intraepithelial nerve fiber endings on top of a papillae very often show spiny structures (arrowhead). The intrapapillar enlargement (arrow) from which the branches stretch up into the epithelium is also visible. x 1,520

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Regarding the organization of nerve fibers in the subepithelial connective tissue, it has to be emphasized that delicate anastomoses seemed to exist between nerve fiber branches. In this context, it should be mentioned that it was not possible to decide whether the anastomoses connect the branches of one neuron or two

neurons; the latter mentioned alternative is not very likely because it would contradict the neuron-theory.

An additional interesting result was the demonstration of the detailed morphology of the papillar nerve fiber plexus. In the present study, it could be shown that these nerve fiber complexes expanded into

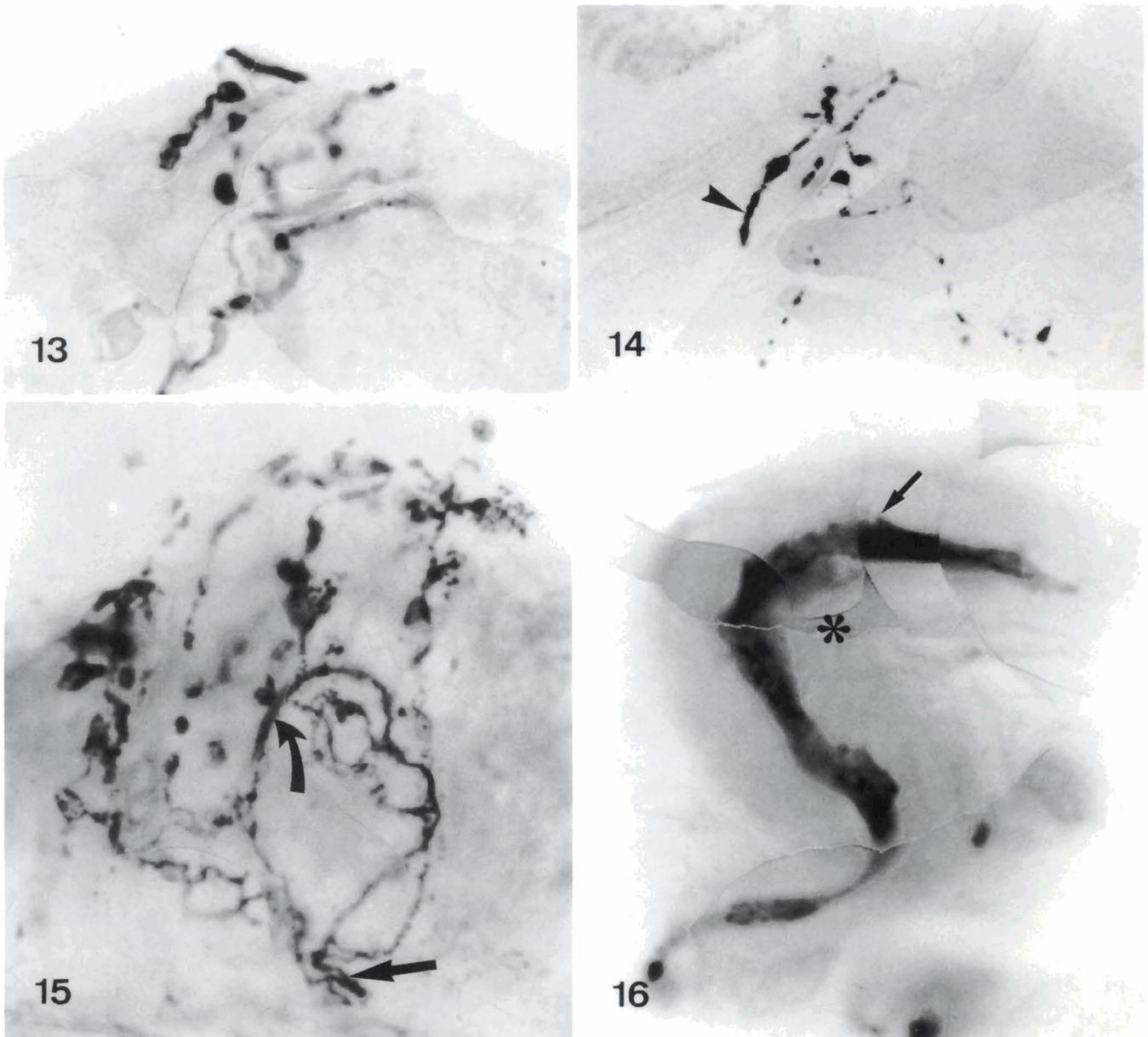


Fig. 13. Palatoglossal arch. The terminations of the intraepithelial nerve fiber branches exhibit polymorphic features. x 1,230

Fig. 14. Palatoglossal arch. Lanceolate nerve endings (arrowhead) could also be found. x 1,230

Fig. 15. Soft palate. Very often, a single intrapapillar nerve fiber forms a loop (curved arrow) from which branches radiate into the epithelium. The loop can also be regarded as an anastomosis between two branches of a single nerve fiber (arrow). x 1,000

Fig. 16. Palatoglossal arch. Encapsulated thick nerve fiber endings are found. The surrounding capsule is only slightly stained (asterisk). The encapsulated axon shows lateral spiny protuberances (arrow). x 1,600

the epithelium at the top of the papillae. Moreover, it became apparent that differences in configuration of the nerve fiber plexus between the soft palate and the palatoglossal arch exist. Apparently, this MB-stained nerve fiber plexus does not represent a rare type of nerve fiber ending because it was found on top of nearly all connective tissue papillae.

Moreover, the intensely stained unbranched nerve fiber endings which were enclosed by a slightly stained capsule might belong to the simple type of encapsulated sensory corpuscles, as was observed by other investigators (Malinovsky, 1966; Yamamoto et al., 1986; Tachibana et al., 1987).

In addition, van der Werf et al. (1980, 1982) proved electron microscopically in the hard palate of the rat that intrapapillar nerve fiber endings resemble the lanceolate

endings which are palisade-like arranged around hair follicles and therefore represent mechanoreceptors. Interestingly, the existence of lanceolate endings could also be demonstrated in a few nerve fiber plexus in the present study. These results are in accordance with former observations obtained by silver impregnation (Gairns, 1955). However the other polymorphic types of terminal enlargements might also fulfill mechanoreceptive functions.

A further important finding was the staining of Merkel's discs and their associated nerve fibers in both investigated parts of the oral mucosa. This observation is in full accordance with those of other investigators (Botezat, 1901; Yamamoto et al., 1986). Furthermore, the nerve fiber branches ending as Merkel's discs appeared to be interconnected by anastomoses.

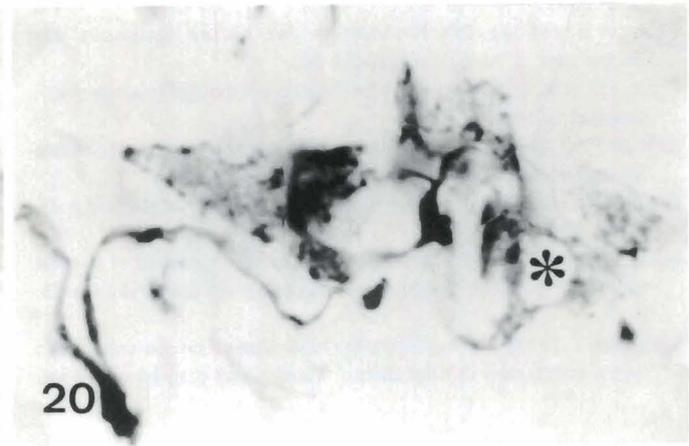
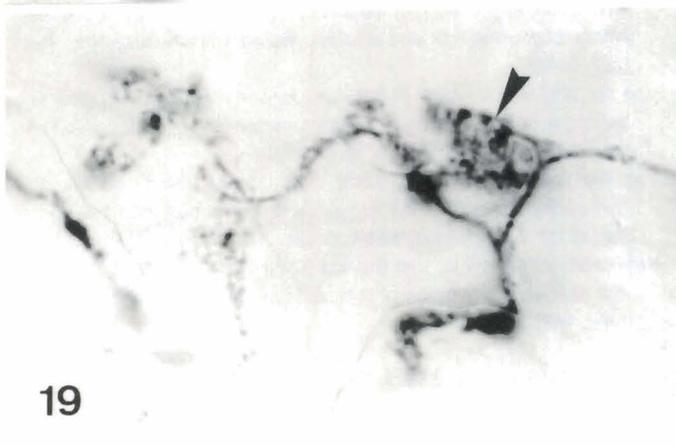
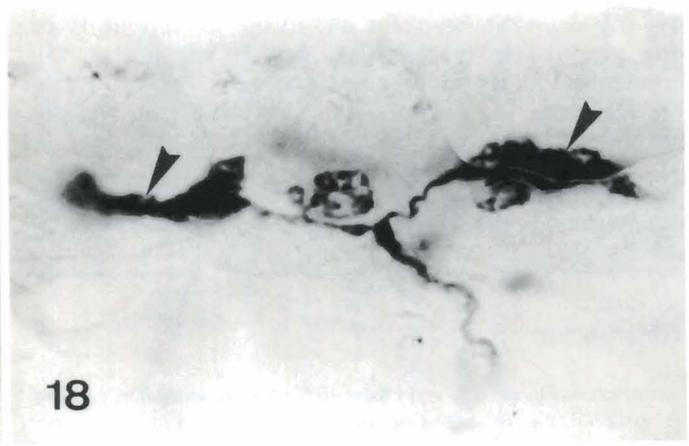
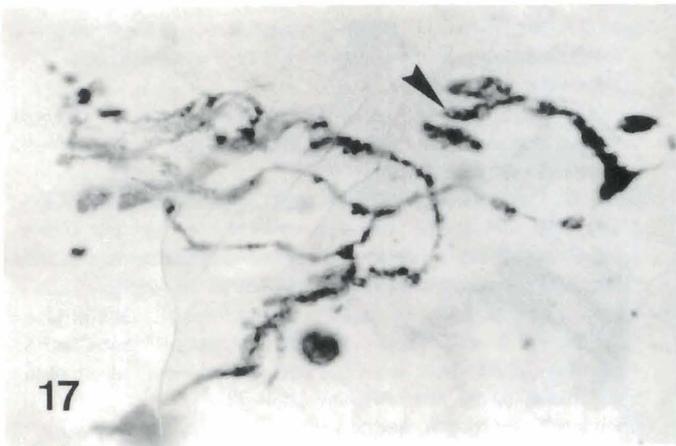


Fig. 17. Soft palate. At the base of the epithelia, nerve fiber plexus are found ending with characteristic disc-like enlargements (arrowhead). These enlargements appear to represent Merkel's discs. x 970

Fig. 18. Soft palate. In the higher magnification of longitudinal sections, the typical morphology of Merkel's discs (arrowheads) can be studied in detail; the Merkel cells themselves are not stained. x 1,800

Fig. 19. Palatoglossal arch. In transverse section, Merkel's discs seem to represent a kind of enlarged anastomosis (arrowhead) between the branches of a nerve fiber. x 1,600

Fig. 20. Palatoglossal arch. The higher magnification reveals that the Merkel's discs and interconnecting nerve fiber branches form a terminal network. A single mesh (asterisk) of the net can clearly be identified. x 1,950

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Therefore, Merkel's discs have to be regarded as components of a terminal network of nerve fiber branches probably belonging to one neuron. In this context, it has to be pointed out that MB-staining reveals more details than vital staining with styryl pyridinium fluorescent dyes (Nurse and Faraway, 1989). The lack of MB-staining of Merkel cells supports the hypothesis that these cells are of epithelial and not of neural crest origin (Moll et al., 1984); supravital MB-uptake is a characteristic property of neurons and sensory cells (Ehrlich, 1886).

In conclusion, due to its high selectivity for nerve fiber terminations a complete mapping of the innervation pattern of the whole oral mucosa using MB-staining becomes necessary. Due to the low costs of the dye's use, large pieces of tissues can be stained and serial sections can be made. Therefore, the demonstrated modification of the MB-staining technique offers manifold possibilities for use in the investigation of peripheral innervation patterns. In particular, the described method might be useful for studying alterations in nerve fiber distribution during development, aging or disease.

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References

- Arvidsson J., Fundin B.T. and Pfaller K. (1995). Innervation of the hard palate in the rat studied by anterograde transport of horseradish peroxidase conjugates. *J. Comp. Neurol.* 351, 489-498.
- Botezat E. (1901). Die Innervation des harten Gaumens der Säugethiere. *Z. Wiss. Zool.* 69, 429-443.
- Dixon A.D. (1961). Sensory nerve terminations in the oral mucosa. *Arch. Oral Biol.* 5, 105-114.
- Ehrlich P. (1886). Ueber die Methylenblaureaction der lebenden Nervensubstanz. *Dtsch. Med. Wochenschr.* 12, 49-52.
- Gairns F.W. (1955). The sensory nerve endings of the human palate. *J. Exp. Physiol.* 40, 40-48.
- Halata Z. and Munger B.L. (1983). The sensory innervation of primate facial skin. II. Vermilion border and mucosa of lip. *Brain Res. Rev.* 5, 81-107.
- Itotagawa T. (1990). Immunohistochemical studies on the peptidergic nerve distribution in hard palate mucosa and gingiva of the rat. *Osaka Daigaku Shigaku Zasshi* 35, 78-92.
- Kadanoff D. and Gürowski A. (1963). *Morphologie der Rezeptoren des Atmungs- und Verdauungssystems beim Menschen.* Verlag Gustav Fischer. Jena.
- Malinovsky L. (1966). The variability of encapsulated corpuscles in the upper lip and tongue of the domestic cat (*Felis ocreata* L. f. *domestica*). *Folia Morphol.* 14, 175-191.
- Moll R., Moll I. and Franke W.W. (1984). Identification of Merkel cells in human skin by specific cytokeratin antibodies: changes of cell density and distribution in fetal and adult plantar epidermis. *Differentiation* 28, 136-154.
- Müller T. (1990). Supravital staining of murine brain with methylene blue according to the Cajal method: a simple and reliable preparation technique for embedding in paraffin. *J. Neurosci. Methods* 32, 223-226.
- Müller T. (1994). Large nerve cells with long axons in the granular layer and white matter of the murine cerebellum. *J. Anat.* 184, 419-423.
- Nurse C.A. and Faraway L. (1989). Characterization of Merkel cells and mechanosensory axons of the rat by styryl pyridinium dyes. *Cell Tissue Res.* 255, 125-128.
- Ogawa H., Hayama T. and Ito S. (1982). Convergence of input from tongue and palate to the parabrachial nucleus neurons of rats. *Neurosci. Lett.* 28, 9-14.
- Ramieri G., Panzica G.C., Viglietti-Panzica C., Modica R., Springall D.R. and Polak J.M. (1992). Non-innervated Merkel cells and Merkel-neurite complexes in human oral mucosa revealed using antiserum to protein gene product 9.5. *Arch. Oral Biol.* 37, 263-269.
- Rodrigo J., Polak J.M., Terenghi C., Cervantes C., Ghatei M.A., Mulderry P.K. and Bloom S.R. (1985). Calcitonin gene-related peptides (CGRP)-immunoreactive sensory and motor nerves of the mammalian palate. *Histochemistry* 82, 67-74.
- Tachibana T., Ishizeki K. and Sakakura Y. (1987). Distinct types of encapsulated sensory corpuscles in the oral mucosa of the dog: immunohistochemical and electron microscopic studies. *Anat. Rec.* 217, 90-98.
- Van der Werf A.A.D., Liem R.S.B. and Jansen H.W.B. (1980). The fine structure of nerve fibres in the hard palate of the Wistar albino rat. *Acta Morphol. Neerl. Scand.* 18, 273-280.
- Van der Werf A.A.D., Liem R.S.B. and Jansen H.W.B. (1982). The innervation pattern of the rugae on the hard palate of the Wistar Albino rat. *Acta Morphol. Neerl. Scand.* 20, 283-292.
- Yamamoto T., Tazaki M. and Sakada S. (1986). Morphological study of the sensory innervation of the rat labial mucosa. *Tohoku J. Exp. Med.* 148, 229-238.

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