Nerve fibers immunoreactive to calcitonin gene-related peptide, substance P, neuropeptide Y, and dopamine β-hydroxylase in innervated and denervated oral tissues in ferrets

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The effect of sensory and sympathetic denervation on the localization and distribution of nerve fibers immunoreactive (IR) to calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and dopamine β-hydroxylase (DBH) was studied in the dental pulp, periodontal ligament (PDL), and gingiva in ferrets. Unilateral axotomy was performed by resection of the inferior alveolar nerve (IAN) 10 days before the experiment (Group 1); sympathectomy, by unilateral removal of the cervical ganglion 5 days before the experiments (Group 2). Immunohistochemistry was performed on free-floating sections by the avidin–biotin–peroxidase technique. A considerably higher density of sensory fibers IR to CGRP and SP was found in the dental pulp than in PDL and gingiva. The majority of pulpal fibers were located in the walls of blood vessels. A subodontoblastic network of fibers IR to CGRP and SP was lacking in incisors and canines and was found only in the coronal pulp in premolars and molars. Sympathetic fibers were sparsely distributed in the pulp, and they were mainly confined to large vessels running centrally in the root pulp as well as the larger vessels in apical PDL and alveolar bone. Gingiva was well supplied with CGRP- and SP-IR nerves, and some NPY and DBH fibers were located in association with larger vessels. Round cell-like structures within the basal part of the epithelium were CGRP-IR. Axotomy induced a complete loss of CGRP- and SP-IR fibers in the anterior part of the jaws, whereas sympathectomy caused a reduction, but not a total loss, of NPY- and DBH-IR nerves. It is concluded that, except for some distributional differences, the oral tissues in the ferret have an abundant sensory innervation similar to that found in other species. □ *Axotomy; dental pulp; neuropeptide; neurotransmitter; sympathectomy*

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Nerve fibers in teeth and periodontium have previously been mapped by use of various techniques in several species, including man (1, 2), cat (3–6), rat (7, 8), and mouse (9). So far nobody has described the peripheral nerves and verified their origin in this area in the ferret by use of immunohistochemistry. This animal (*Mustela putorius furo*) is commonly used as a research animal and has a non-rodent dentition with three incisors, one canine, three premolars, and two molars in each quadrant.

In a recent study (10) we have shown that a resting nervous vasodilator tone of sensory origin seems to exist in the ferret dental pulp. Furthermore, we found that the sensory nerves are responsible for an increase in pulpal blood flow and interstitial fluid pressure during electrical tooth stimulation, whereas sympathectomy had no effect on blood flow (resting or not) or interstitial fluid pressure during tooth stimulation (10).

In the present study we therefore wanted to verify whether the ferret dental tissues have the same abundant supply and distribution of nerves immunoreactive (IR) to the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) as described in other species. Moreover, to identify the localization and distribution of sympathetic fibers, antibodies directed against neuropeptide Y (NPY) and dopamine β-hydroxylase (DBH) were used. NPY and noradrenaline (NA) have frequently been shown to coexist in perivascular sympathetic fibers in oral tissues from several species. NPY is regarded as the mediator of the non-adrenergic vasoconstriction observed when sympathetic perivascular nerves are stimulated (11–14). Antibody against DBH was used as a marker for catecholaminergic nerves (15, 16). To determine the origin of the different IR nerve fibers, unilateral surgical denervation of the inferior alveolar nerve (IAN) or sympathectomy was performed. Extirpation of the superior cervical ganglion is known to abolish the immunoreactivity to NPY in oral tissues (17, 18) and to reduce the number of catecholaminergic nerves consistently.

Materials and methods

A total of 17 young ferrets (11 female, 6 male; 1–2.3 kg body weight) were anesthetized with 1 mL/kg body weight ketamine hydrochloride (1 mg/mL) mixed with 0.1 mL/kg body weight medetomidin hydrochloride (50 mg/mL) administered intramuscularly. The animal experiments were approved at the University of Bergen under supervision of the Norwegian Experimental Animal Board. The ferrets were divided into two groups. In Group 1 ($n = 8$)

Table 1. Distribution and relative frequency of nerve fibers immunoreactive to calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and dopamine β-hydroxylase (DBH) in various locations in the examined tissues*

		Periodontal ligament		Gingiva	
	Pulp	Apical	Cervical	Epithelium	Connective tissue
CGRP	++++	+++	++	+	+++
SP	++++	+++	++	+	++
NPY	+	++	+	0	+
DBH	++	+++	++	0	++

* Semiquantitative estimations: ++++, very many nerve fibers; +++, many fibers; ++, moderate number of fibers; +, few fibers; 0, no fibers detected.

unilateral surgical denervation of the IAN was performed through an intraoral incision bucco inferior behind the last molar on the left side of the mandible. The soft tissue and periost were retracted, and the mandibular bone was made visible. The IAN was thereafter exposed by drilling a cavity with a round dental bur (ISO nr 310.204.001001.023, Komer, Germany) in a slow-speed hand piece, cooled with sterile saline. To avoid reinnervation during the experiment, approximately 3 mm of the nerve was carefully removed, without damaging the blood vessels. The wound was closed with two or three sutures in the vestibular mucosa. In Group 2 ($n = 9$) unilateral sympathectomy was performed by surgical removal of the left superior cervical ganglion. The sympathetic nerve in the neck of the ferret runs together with the vagus in a common vago-sympathetic trunk. Careful dissection of the trunk was necessary to localize the sympathetic nerve, which was then followed in cranial direction until the ganglion was revealed. The left superior cervical ganglion was removed by a vascular scissor, and the neck wound was closed by three to five sutures in the skin. The success of the sympathectomy was indicated by ptosis of the ipsilateral eyelid. An antibiotic, Streptocillin vet. (Boehringer Ingelheim, Ingelheim am Rhein, Germany), 1 mL/kg body weight, and an analgesic, Temgesic (Reckitt & Colman), 0.3 mL/kg body weight, were administered intramuscularly after denervation in both groups. The observation time was 10 days in Group 1 and 5 days in Group 2. At the end of the observation periods all animals were deeply reanesthetized with an overdose of Ketalar/Medetomidin. Transcardiac perfusion was done with phosphate buffered saline (PBS) containing 0.003% Heparin followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The jaws were excised, postfixed for 24 h, and demineralized in 4 N formic acid and 0.05 M sodium formate at 4°C for 2–3 weeks. In Group 1 the lower jaws were excised, and in Group 2 all four jaws were excised.

Immunohistochemistry

After demineralization the specimens were rinsed in PBS for 24 h and saturated in 30% sucrose in 0.1 M phosphate buffer for another 24 h. The jaws were serially sectioned in a sagittal plane at 40 µm on a freezing microtome. The 40-mm sections were free-floating in tissue culture wells and reacted for visualization of IR fibers. Alternate serial sections from both control and denervated jaws were incubated for 72 h with polyclonal antibody to rat CGRP (1:6000 dilution), SP (1:5000 dilution), NPY (1:4000 dilution; Cambridge Research Biochemicals, Cambridge, UK), or DBH (1:4000 dilution; Biogenesis Ltd., Poole, UK). The antigen–antibody complex was localized by the avidin–biotin–peroxidase reaction, using a commercially available ABC kit (Vectastain ABC kits, Vector Laboratories Inc., Burlingame, Calif., USA) and visualized by means of 3′3 diaminobenzidine (Sigma Chemical Co., St. Louis, Mo., USA) in the presence of 0.2% $(NH_4)_2Ni(S0_4)6H_2O$ to enhance the chromogen staining.

After several rinses in PBS, the sections were mounted on gelatin-coated slides, air-dried, and counterstained with methylene blue/azure II in 1% sodium borate and distilled water. Thereafter, they were dehydrated in graded alcohols, cleared in xylene, and coverslipped with Eukitt (O. Kindler, Freiburg, Germany). Immunocontrols were routinely performed, either by preabsorption of the primary antibody with its antigen before incubation or by replacement of the primary or secondary antibody with PBS. The controls did not show any immunolabeling.

Evaluation

Observations and microphotography were done with a Leitz light microscope at ×25 to ×400. The semiquantitative evaluations of nerve fibers IR to SP, CGRP, NPY, and DBH were done independently by two investigators for at least two sections for each antibody from all teeth. The sections were coded to conceal their identity. The experimental side was consistently compared with the contralateral control side to avoid interindividual differences.

Results

Dental pulp

A considerably higher density of nerve fibers IR to CGRP and SP was found in the dental pulp than in the

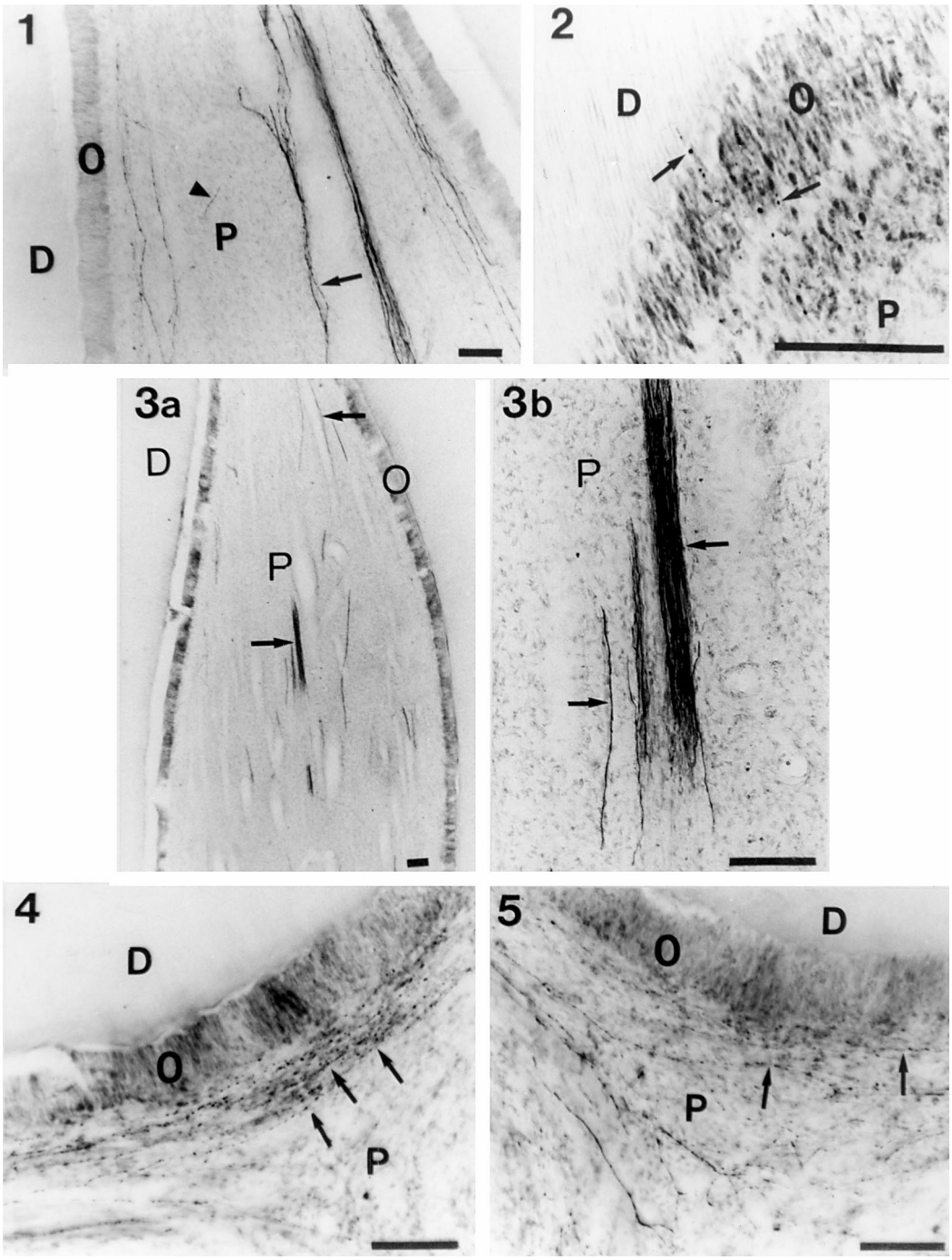

Fig. 1. Fibers immunoreactive to calcitonin gene-related peptide in the pulpal stroma with (arrows) and without (arrowheads) connection to blood vessels. Note the lack of fibers in the odontoblastic layer (O). Section from canine pulp, control side, Group 1. D, dentin; P, pulp. Fig. 2. Section from a control canine, Group 1, incubated with calcitonin gene-related peptide antibody. Nerve fibers (arrows) run into the dentin (D) without forming a subodontoblastic plexus. O, odontoblast layer; P, pulp. Fig. 3a. Longitudinal section of a control canine in the lower jaw immunolabeled with antibody against substance P. D, dentin; P, pulp; O, odontoblast layer. 3b. Higher magnification of outlined area in 3a showing centrally running fibers immunoreactive to substance P (arrows) in the pulp (P). Fig. 4. Immunohistochemical micrograph demonstrating fibers immunoreactive to substance P forming a subodontoblastic plexus (arrows) in the coronal part of a control third premolar, Group 2. D, dentin; O, odontoblast layer; P, pulp. Fig. 5. Extensive branching of fibers immunoreactive to calcitonin gene-related peptide (arrows) beneath the odontoblast layer (O) in the coronal pulp (P) of a control molar, Group 2. D, dentin. Bars (Figs. 1–5) = 100 μm.

surrounding tissues, including periodontal ligament (PDL), gingiva, and alveolar bone (Table 1). In the innervated control side all pulps were densely supplied with SP- and CGRP-IR fibers (Figs. 1 and 3). The distribution and density of these two neuropeptides were about equal, but the SP-IR fibers usually appeared thinner and were more faintly stained than the CGRP-IR fibers. The majority of IR fibers were located in the central pulps mainly associated with the walls of blood vessels (Figs. 1 and 3). However, we also observed fibers that apparently had no connection to blood vessels. IR fibers were rarely observed in the subodontoblast or the odontoblast layer in incisors and canines (Figs. 1 and 3). Sporadically, a few fibers could be traced in these areas, occasionally reaching the innermost part of dentin in the coronal pulp (Fig. 2). These fibers usually arose from central bundles, proceeding through the odontoblastic layer, sometimes extending into dentin, without forming a distinct subodontoblastic plexus. A sensory subodontoblastic network of IR fibers was exclusively found in the coronal pulp of premolars and molars (Figs. 4 and 5). In these teeth CGRP- and SP-IR fibers more frequently penetrated the odontoblast layer, advancing into the innermost part of dentin (Fig. 6). The IR fibers were measured to extend maximally 100 μm into dentin.

In contrast to the abundant presence of CGRP and SP fibers, sympathetic fibers IR to DBH and NPY were sparsely distributed in the pulps of all teeth (Table 1). As a rule, DBH-IR fibers were traced in nerves located in the central part of the pulp in connection with blood vessels, but could also be observed as free fibers in the pulpal stroma (Fig. 7). The number of DBH-IR fibers was highest in the apical part of the pulp, and fibers were never observed in the odontoblast layer or dentin. The density of DBH-IR fibers was consistently less than that observed for fibers IR to CGRP and SP. The distribution of NPY fibers was even more sparse than the distribution of DBH-IR fibers. Most pulps were devoid of NPY fibers, and when present they were mainly confined to centrally running, large vessels in the apical part of the pulp (Figs. 8a,b). These fibers extended about two-thirds into the root pulp (Fig. 8a) and were never observed in the coronal part. After unilateral sympathectomy (Group 2) or axotomy (Group 1), hardly any NPY fibers were found in the pulps. Sympathectomy and axotomy caused a reduction of DBH-IR nerve fibers as well, but not a total loss.

Axotomy (Group 1) caused complete loss of CGRP- and SP-IR fibers in the incisors, canines, and premolars (Fig. 10), whereas a reduction of nerve fibers was observed in the first molar (Fig. 9). In the second molar there were no observed differences in CGRP and SP fibers compared with the innervated contralateral control side. Unilateral sympathectomy (Group 2) caused no change in density or distribution of pulpal CGRP- and SP-IR fibers.

Gingiva

Fibers IR to CGRP appeared more frequently than SP-IR fibers in the submucosa (Table 1). Most of the nerve fibers IR to CGRP and SP ran parallel to the epithelial surface (Fig. 11). Occasionally, fibers were found to penetrate into the epithelium (Figs. 11 and 12). However, SP fibers were regularly observed in the epithelium lining the gingival pocket, sometimes extending through the epithelium (Fig. 13). There were only a few NPY-IR nerve fibers in the gingiva (Table 1). When present, they were located merely in the submucosa associated with larger vessels (Fig. 14). The location of DBH-IR nerve fibers was similar to that of NPY-IR fibers, but they were observed more frequently (Fig. 15). Neither DBH- nor NPY-IR fibers were located within the gingival epithelium (Table 1). Both sympathectomy and axotomy caused a consistent reduction of these fibers, but not a complete loss.

In some areas of the basal layer of the rete pegs, round cell-like structures regularly showed CGRP immunoreactivity (Figs. 11 and 16). The IR cells were round or ovoid, and usually located in clusters (Fig. 16). Thin varicose CGRP-IR fibers were at times observed to contact the cell-like structures (Fig. 17). These cells were distributed equally on the denervated and the contralateral innervated side in both groups. In contrast, SP- and CGRP-IR nerve fibers were almost lacking in the anterior part of the gingiva after axotomy, whereas sympathectomy (Group 2) did not affect the existence of these fibers.

Periodontal ligament

Nerve fibers IR to SP and CGRP entered the periodontal ligament (PDL) from both apical and lateral bone. The periapical PDL was extensively innervated (Figs. 18a,b), whereas in the midroot and cervical parts IR fibers occurred more scarcely (Fig. 19, Table 1). Most CGRP- and SP-IR fibers were associated with blood vessels (Figs. 18 and 19). The larger vessels in the apical half of the PDL (Fig. 20) and alveolar bone (Fig. 21) were regularly supplied with NPY-IR nerves. Otherwise, NPY-IR fibers were rarely observed in the cervical part of the PDL. DBH fibers were found in all parts of the PDL (Fig. 22), with the highest density in the apical area. Neither DBH nor NPY nerve fibers in the PDL were completely lost after removal of the superior cervical ganglion (Group 2).

Discussion

The present immunohistochemical study provides clear evidence of an abundant CGRP- and SP-IR nerve supply in the ferret dental tissues. The vast majority of those fibers were located in the walls of blood vessels, indicating that they may participate in regulation of blood flow and vascular permeability. The dental pulp showed a considerably higher density of CGRP- and SP-IR fibers than the PDL, gingiva, and alveolar bone. However, contrary to observations in other species (e.g. cat, human), scarcely any network of these fibers was located in the odontoblast and subodontoblast areas in the incisors and the canines.

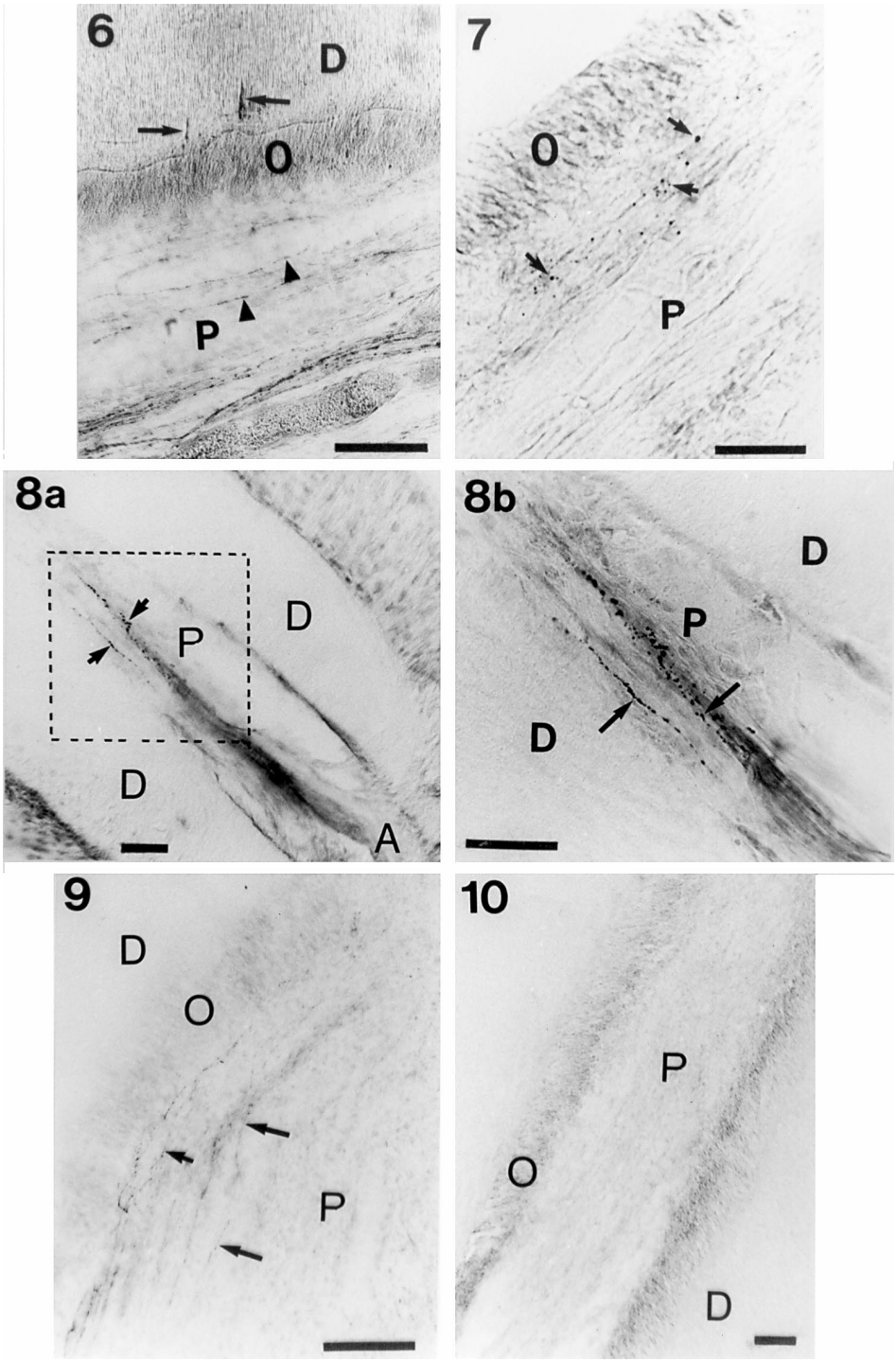

Fig. 6. Nerve fibers immunoreactive to substance P (arrows) in the inner part of dentin (D) in the coronal pulp of a control molar. Thin fibers (arrowheads) forming a subodontoblastic plexus in the pulp (P). O, odontoblast layer. Fig. 7. Dopamine β-hydroxylase immunoreactivity (arrows) without any observable connection to blood vessels in the coronal pulp (P) of an incisor. O, odontoblast layer. Fig. 8a. Apical area (A) of a control incisor immunoreacted against neuropeptide Y (NPY). NPY fibers (arrows) are seen in the wall of a larger vessel. 8b. Higher magnification of outlined area in 8a. D, dentin; P, pulp. Fig. 9. Section from an experimental first molar, Group 1. Fewer nerve fibers (arrows) immunoreactive to calcitonin gene-related peptide remain in the pulp (P) after axotomy. D, dentin; O, odontoblast layer. Fig. 10. Substance P–immunoreacted section demonstrating a complete lack of immunoreactive fibers in an incisor after axotomy, Group 1. O, odontoblast layer; P, pulp; D, dentin. Bars (Figs. 6–10) = 100 μm.

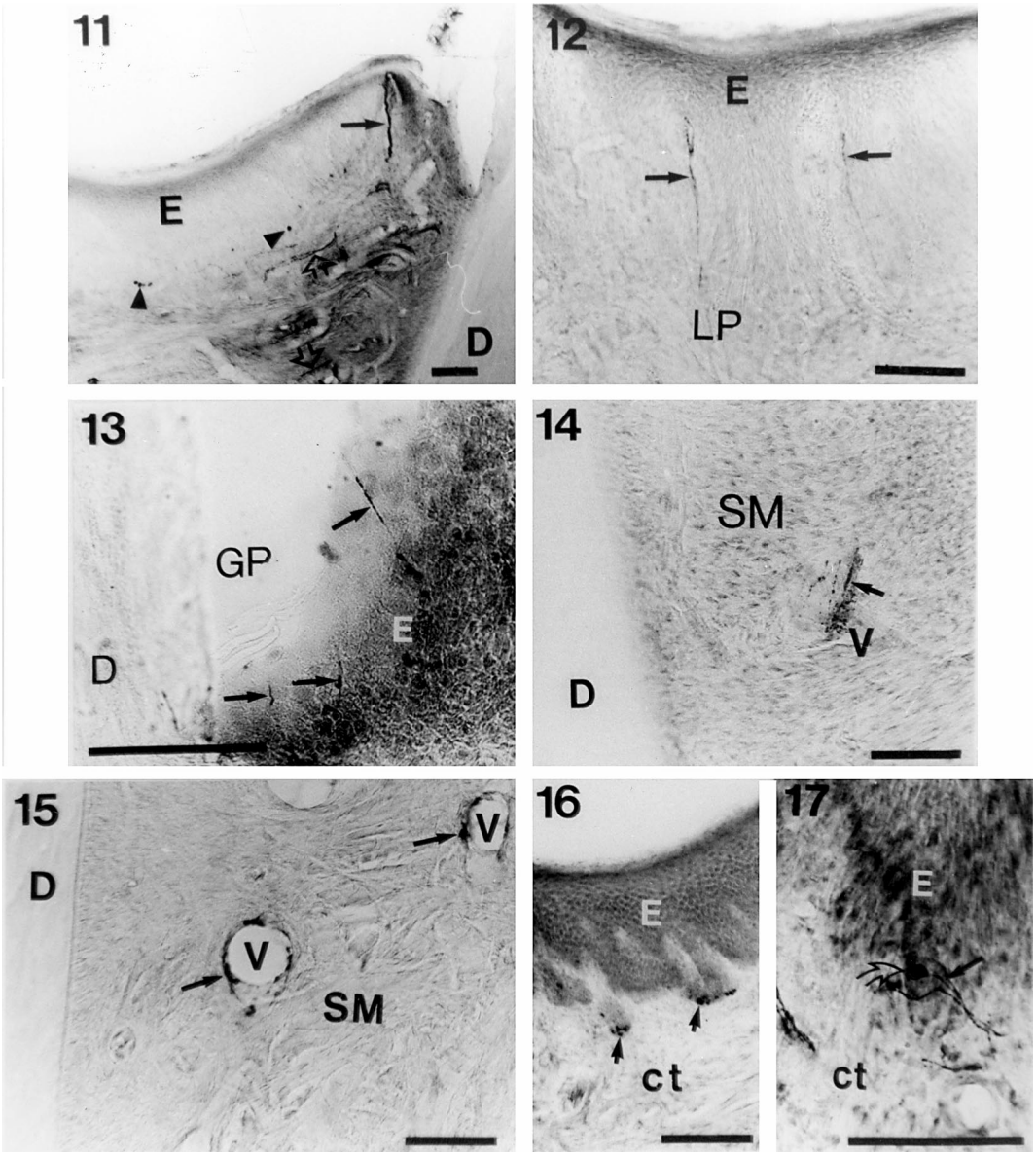

Fig. 11. Nerve fibers immunoreactive to calcitonin gene-related peptide (wide arrows) in lamina propria and submucosa paralleling the epithelial surface of gingiva anterior to a first molar, control side. A nerve fiber (arrow) penetrates the epithelium (E). Immunoreactive cells (arrowheads) are located in the basal layer of the rete pegs. D, dentin. Fig. 12. Thin substance P–immunoreactive fibers (arrows) reaching into the epithelial layer (E). Section from the control side. LP, lamina propria. Fig. 13. In the gingival pocket (GP), substance P–immunoreactive nerve fibers (arrows) were frequently observed. Some fibers extend throughout the epithelium (E) to the surface. D, dentin. Fig. 14. Micrograph from gingiva on control side, Group 2, showing neuropeptide Y–immunoreactive fibers in close connection to a blood vessel (V) in the submucosa (SM). D, dentin. Fig. 15. Longitudinal section of the gingiva, control side (Group 2), immunoreacted for dopamine β-hydroxylase (DBH). DBH-immunoreactive fibers (arrows) are traced in the walls of blood vessels (V). D, dentin; SM, submucosa. Fig. 16. In the basal layer of the epithelium (E), clusters of cell-like structures immunoreactive to calcitonin gene-related peptide (arrows) are observed. Section from gingiva on the control side (Group 1). ct, connective tissue. Fig. 17. Calcitonin gene-related peptide–immunolabeled nerve fiber (arrow) in contact with an immunoreactive cell-like structure (wide arrow) in the mesial gingiva of a control first molar (Group 2). E, epithelium; ct, connective tissue. Bars (Figs. 11–17) = 100 μm.

Only occasionally CGRP- and SP-IR fibers penetrated the odontoblastic layer, reaching the innermost part of dentin in single-rooted teeth, whereas the pulp of premolars and molars exhibited a relatively dense subodontoblastic network of CGRP- and SP-IR fibers, which frequently penetrated the innermost part of dentin. The functional effect of a lacking odontoblast/subodontoblast network of CGRP- and SP-IR nerve fibers in the front teeth and canines is not readily explained. In the rat incisor the pulpal axons are seen centrally, and they do not form a

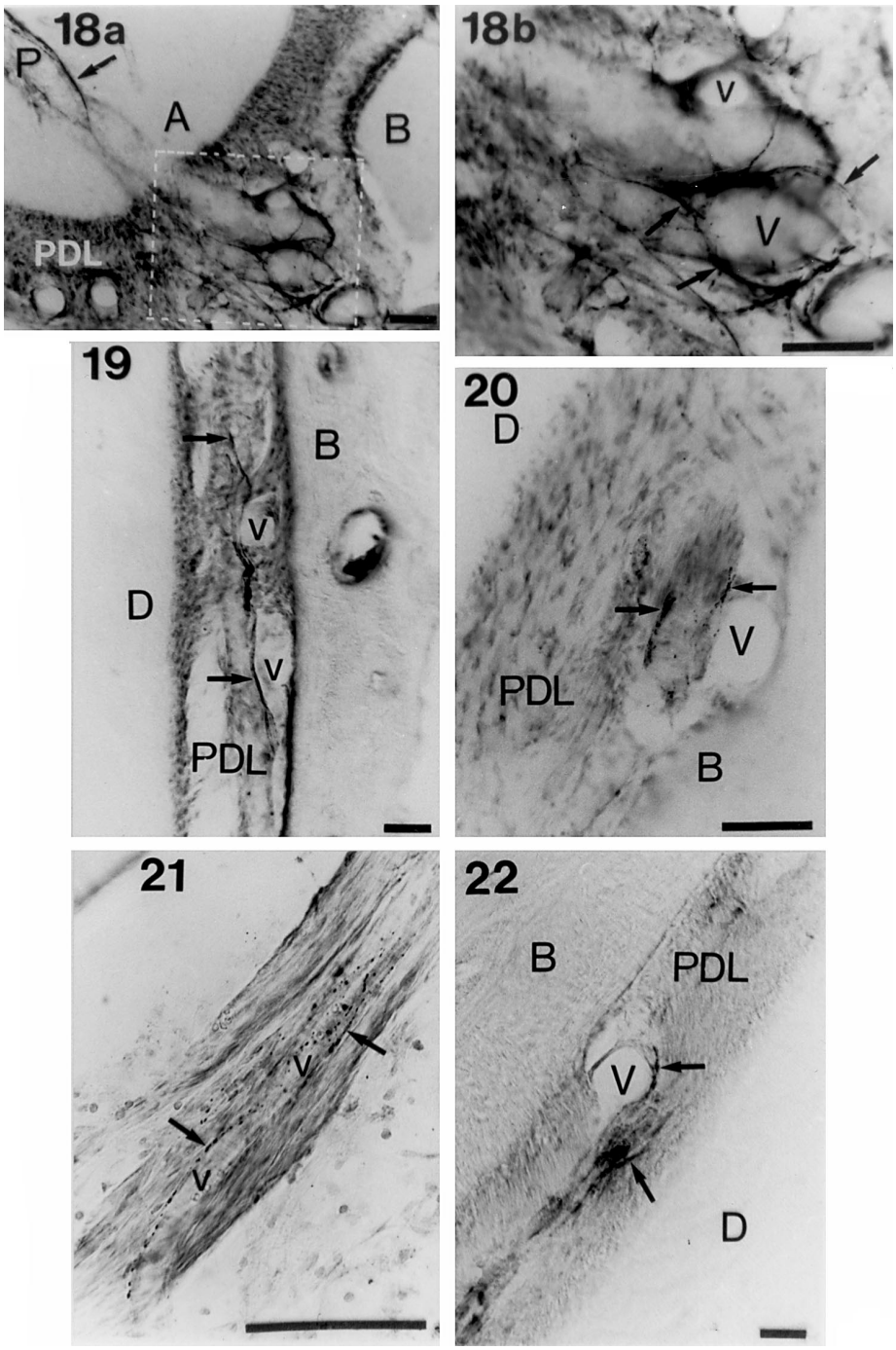

Fig. 18a. Section showing the root apex (A) of a canine, control side, Group 1. Calcitonin gene-related peptide (CGRP) fibers (arrow) enter the pulp (P) from the periapical area (outlined). PDL, periodontal ligament; B, bone. 18b. Outlined area from 18a with an abundant supply of CGRP-immunoreactive fibers (arrows) in close proximity to blood vessels (V). Fig. 19. Longitudinal section from the cervical part of the periodontal ligament (PDL) demonstrating substance P–immunoreactive fibers (arrows) running along with blood vessels (V). Control side, Group 1. D, dentin; B, bone. Fig. 20. Neuropeptide Y–immunolabeled section from the apical half of the periodontal ligament (PDL), control side, Group 2. Thin immunoreactive fibers (arrows) are observed close to blood vessels (V). D, dentin; B, bone. Fig. 21. Vessel (V) in the alveolar bone richly supplied with varicose nerve fibers immunoreactive to neuropeptide Y (arrows). Section from the control side, Group 1. Fig. 22. Dopamine β-hydroxylase–immunoreactive fibers (arrows) in the midroot part of the periodontal ligament (PDL), traced in a section from the control side, Group 2. B, bone; V, vessel; D, dentin. Bars (Figs. 18–22) = 100 μm.

plexus of Raschkow (19). But the rat incisor is continuously erupting, and its innervation properties may represent a special case. In the ferret the lack of CGRP- and SP-IR nerve fibers in the odontoblast/subodontoblast area might make these teeth more insensible, owing to the strong mechanical wearing of these teeth when hunting.

There was no obvious change in the distribution of CGRP- and SP-IR fibers after sympathectomy in any of the investigated areas. This is in line with previous studies of feline dental pulp (4), but contrasts with observations of Terenghi et al. (20), who found that perivascular CGRP-IR fibers increased slightly in the oral cavity in rats and guinea pigs 1 month after removal of the superior cervical ganglion. The discrepancy may be due to the difference in species or to different observation periods, indicating that the previously observed increase in CGRP perivascular fibers may take place later than 5 days after sympathectomy.

The density of DBH- and NPY-IR nerve fibers was considerably lower than, and their distribution different from, that of the sensory fibers in all the areas studied. The sympathetic fibers were regularly observed in connection with relatively large vessels, suggesting that sympathetic control of blood flow is mainly confined to the big vessels, agreeing well with previous findings (4, 6, 21–23). In the dental pulp both NPY- and DBH-IR fibers were sparsely distributed, with the highest density in the apical third of the pulp, contradicting earlier findings in the rat incisor pulp (24), but agreeing well with observations in the cat canine (6). However, some DBH immunoreactivity was also observed beneath the odontoblast layer in the coronal pulp without any apparent connection to blood vessels, suggesting a non-vasoactive function. According to their location it might be suggested that these adrenergic fibers could have an inhibitory effect on the release of neuropeptides from sensory nerve endings, as previously proposed by Kerezoudis et al. (25, 26). The number of adrenergic fibers IR to DBH showed a substantial reduction after sympathectomy (Group 2), but these fibers were never completely abolished, corresponding fairly well with observations by Terenghi et al. (19) and Kerezoudis et al. (24). A reduction of NPY-IR fibers, but not a total loss, was also observed after sympathectomy in the present study. Taken together, these findings may indicate that some sympathetic postganglionic fibers in the oral cavity arise from neurons located in the lower cervical ganglions and follow the larger arteries to the oral tissues.

We frequently observed clusters of small (5–10 μm in diameter) CGRP-IR cells localized in the base of the epithelial rete pegs in the gingiva. These cells often seemed to be in close contact with varicose CGRP-IR fibers and had the same appearance and distribution as those found in cats (6) and humans (27, 28). The cells were round and had no processes, and neither axotomy nor sympathectomy had any effect on their distribution or IR staining. Therefore, these cells are most likely capable of producing CGRP themselves. CGRP receptors have previously been shown on the surface of lymphocytes and macrophages (29, 30), and non-neuronal CGRP-IR cells, most likely macrophages or B-lymphocytes, have been reported in lamina propria in the gastric mucosa (31). However, in our study the labeled cells retained their immunoreactivity even 10 days after axotomy, suggesting that the CGRP immunoreactivity is not due to a receptor binding. Given their size, morphology, and localization, it therefore seems more reasonable to assume that the CGRP-IR cells observed in gingiva are Merkel cells, as also suggested in previous investigations (32, 28). On the other hand, the suggestion that these cells may be part of a protective inflammatory defense reaction (31) cannot be completely excluded. Furthermore, the manner in which the CGRP-IR nerve fibers, which were found in close contact with these cells, interact with the cells remains unknown and needs further investigation.

In conclusion, our study demonstrates that the ferret oral tissues, in particular the dental pulp, have an abundant sensory nerve supply. Sympathetic nerve fibers IR to NPY and DBH were present in the dental pulp, gingiva, and PDL, but these fibers were considerably fewer in number and more sparsely distributed than the sensory fibers IR to CGRP and SP. In both innervated and denervated gingiva, some round cell-like structures localized in the base of the epithelial rete pegs consistently showed CGRP immunoreactivity. For exact identification of these IR cells, further studies using confocal or immunoelectron microscopy will be needed.

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