Nerve fibers immunoreactive to protein gene product 9.5, calcitonin gene-related peptide, substance P, and neuropeptide Y in the dental pulp, periodontal ligament, and gingiva in cats

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The distribution patterns of nerve fibers immunoreactive (IR) to calcitonin gene-related peptide (CGRP), substance P (SP), and neuropeptide Y (NPY) in the dental pulp, periodontal ligament (PDL), and gingiva were studied and compared with the complete innervation visualized by antibody to protein gene product (PGP) 9.5 in adult cats. The pulp showed considerably denser nerve supply for PGP 9.5, CGRP, and SP than the periodontal tissues. Most of the pulpal fibers were CGRP-IR, and approximately three to four times more IR fibers were labeled with CGRP than SP. Most fibers in the odontoblast area penetrating into the dentin tubules were CGRP-IR. NPY-IR nerves were mainly observed in connection with the larger blood vessels in pulp and PDL. In the PDL most nerves were localized in the apical third in connection with blood vessels, but CGRP-IR fibers extending close to root cementum were often observed. Immunoreactivity to PGP 9.5 and CGRP was frequently found in cell-like structures in connection with Malassez epithelium in the PDL and in some round epithelial-like cells located in the base of gingival rete pegs. □ *Blood circulation; dentin; neuropeptides; sensory nerves*

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The tooth and its supporting tissues are heavily supplied by sensory nerves from the trigeminal ganglion (1–4) and by a minor contribution from the sympathetic nerves (5–8). Both the sensory and sympathetic fibers contain neuropeptides that have effect on pulpal blood circulation. Thus, in the cat pulp it has been shown that the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) cause vasodilation (9), whereas neuropeptide Y (NPY) synthesized in the sympathetic cell bodies and transported peripherally by axoplasmatic flow causes vasoconstriction (6). In the dental pulp myelinated A fibers are not seen farther peripherally than the cell-free zone (10). However, the profuse ramification of unmyelinated nerves in this area, and also in the vicinity of the odontoblast layer and predentin/dentin, may include the unmyelinated terminal parts of myelinated A fibers (11–13), as stated already 20 years ago by Dahl & Mjør (10). In the rat pulp both myelinated A fibers and unmyelinated C fibers have been shown to contain CGRP (14). In accordance with findings in skin (15), recent studies indicate that both A fibers and possible C fibers are involved in the axon reflex causing a vasodilatory defense reaction in response to pulp injury in the cat (16–18). Most of the nerve fibers entering dentin in rat molars are immunoreactive (IR) to CGRP (14, 19, 20), and several functional studies have shown that it

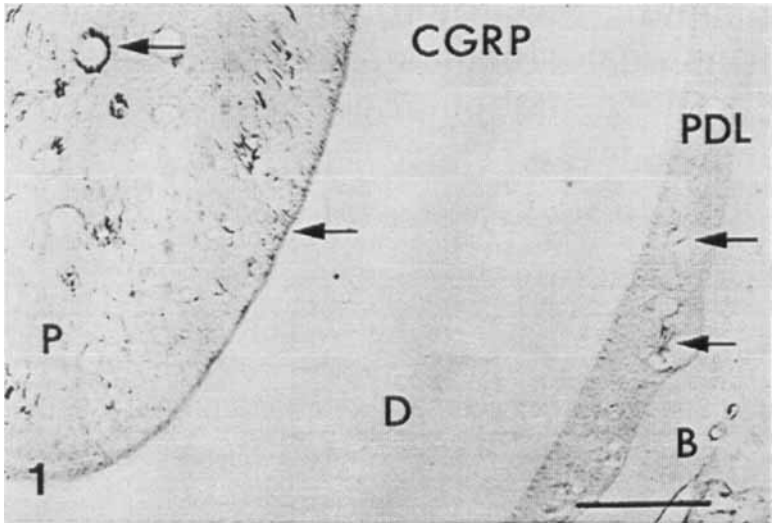


Fig. 1. Horizontal section of canine root pulp (P) and periodontal ligament (PDL), showing calcitonin gene-related peptide-immunoreactive (CGRP-IR) nerve fibers (arrows). D = dentin; B = bone. Bar = 0.5 mm.

is the A fibers that are responsible for the low-threshold dentin sensitivity (for references, see 21, 22).

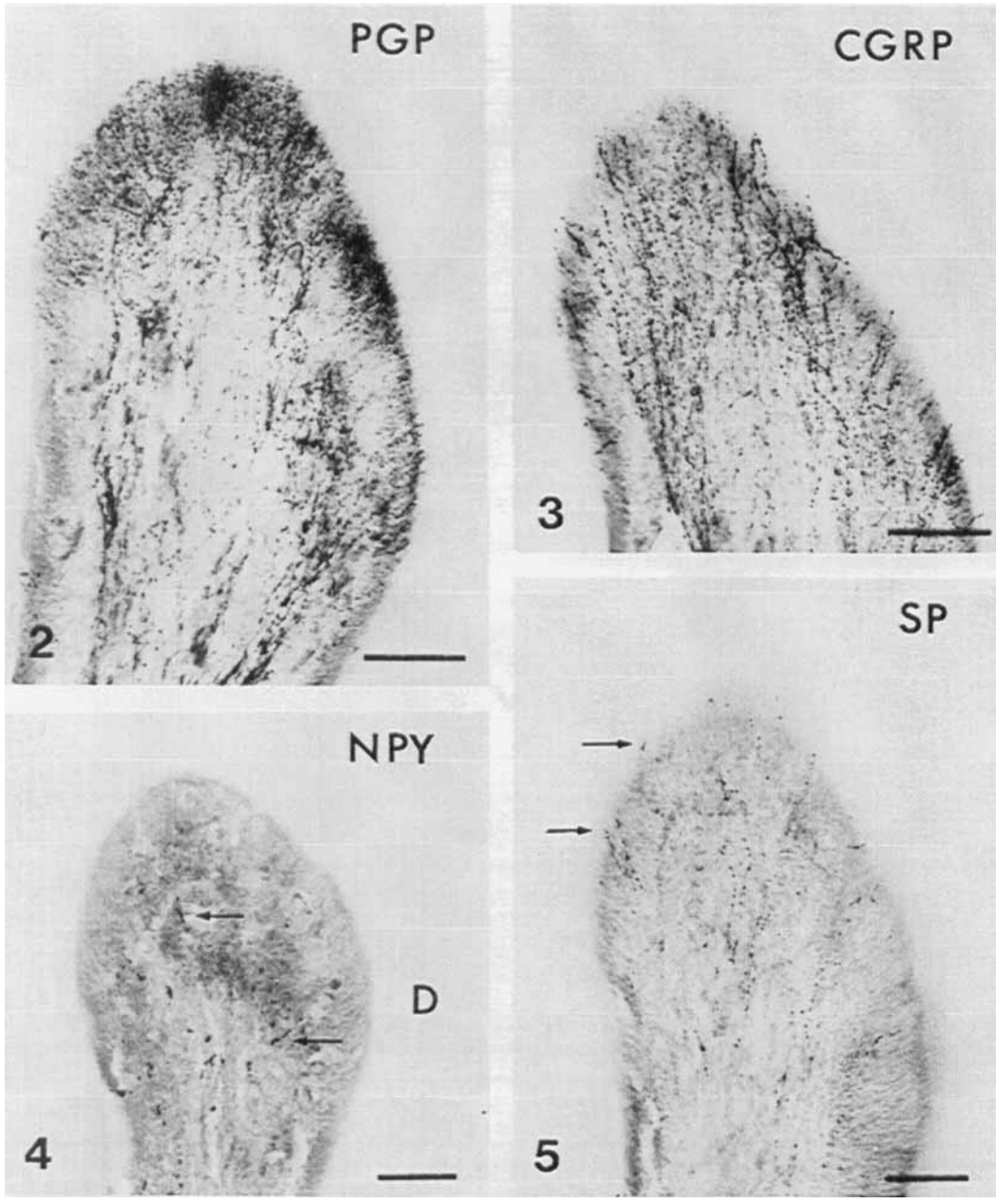
Taken together, these findings might indicate that the A-fibers that are responsible for dentin sensitivity could also release neuropeptides such as CGRP and thus take part in pulpal blood flow regulation via an axon reflex. Accordingly, the nerve fibers in the odontoblast/dentin area might, when excited by the hydrodynamic mechanism (23), initiate an axon reflex by conducting afferent impulses for dentin sensitivity and simultaneously, in efferent direction, cause pulpal vasodilation by releasing CGRP, as already proposed by Matthews (17). Because vasodilation in the low-compliant pulp will increase pulpal tissue pressure (24), it will cause an increased outward flow of fluid through exposed dentin tubules and help protect the pulp against entry of toxins (16, 17). Thus, a neurogenic defense mechanism that helps to protect the pulp might be established when dentin tubules are exposed (17, 25).

However, one prerequisite is that in cat pulp most of the nerves located in the odontoblast, predentin/dentin area are CGRP-IR.

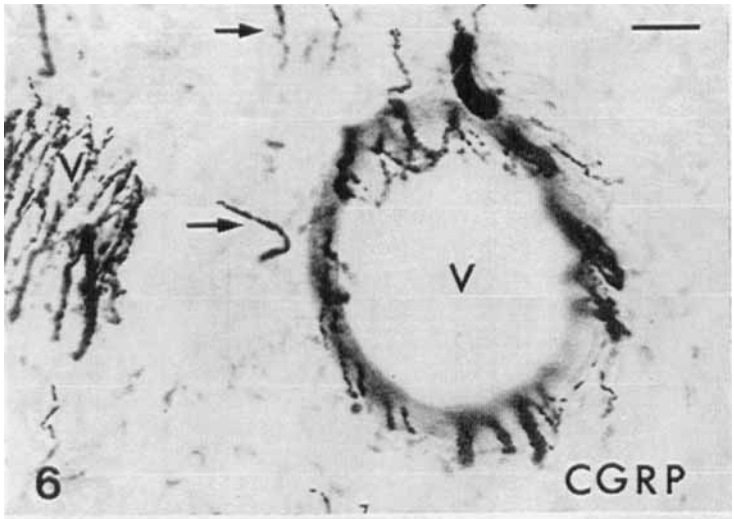
In the present study we therefore wanted to compare the distribution pattern of nerve fibers IR to CGRP, SP, and NPY, to the complete pulpal innervation IR to protein gene product (PGP) 9.5, and, further, to localize the different peptidergic fibers to specific structures in the pulp, periodontal ligament (PDL), and gingiva. As most functional studies of blood flow (24, 26–28) and dentin sensitivity (for references, see 21 and 29) have been performed in cat canines, the distribution pattern of peptidergic nerves in cat canines was given particular attention. A preliminary account of some of these findings has been presented in abstract form (30).

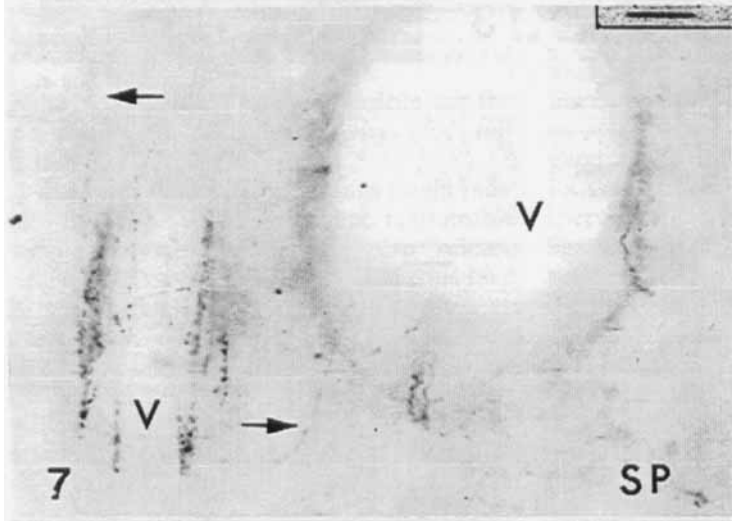
Materials and methods

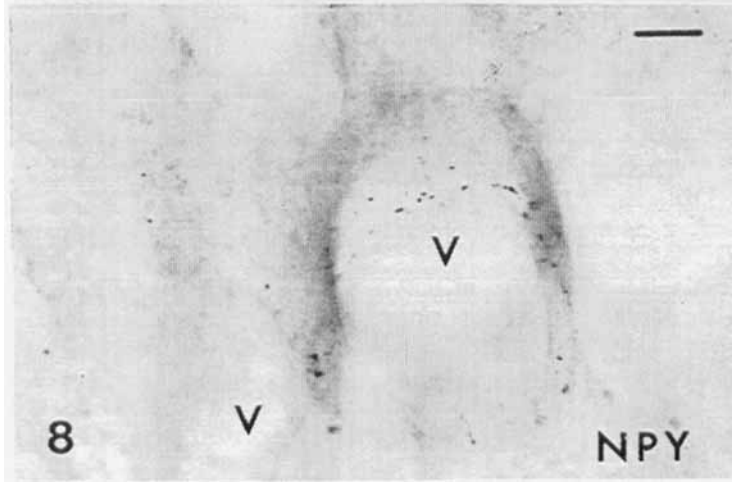
Ten adult cats of both sexes (2.8–3.9 kg body weight) were deeply anesthetized with sodium pentobarbital (30 mg/kg b.w. intraperitoneally) and perfused via the common carotids with heparinized saline followed by Zamboni's fixative (31). The jaws were excised and postfixed in same fixative for 48 h. After demineralization in 4 *N* formic acid and 0.5 M sodium formate at 4°C for 2 weeks, the demineralized specimens were rinsed in phosphate-buffered saline (PBS) for 24 h and saturated in 30% sucrose in 0.1 M phosphate buffer, pH 7.4, for 24 h.



Figs. 2–5. Serial sections from cat incisors immunolabeled with antiserum to protein gene product (PGP) 9.5, calcitonin gene-related peptide (CGRP), substance P (SP), and neuropeptide Y (NPY).
Fig. 2. The complete innervation of pulp and dentin immunoreactive (IR) to PGP 9.5.
Fig. 3. Most of the pulpal nerves are IR to CGRP compared with PGP 9.5-IR nerves.
Fig. 4. Nerves IR to NPY (arrows) are confined to the main pulp; few labeled nerves in pulp periphery. Odontoblast layer and dentin are devoid of NPY-IR fibers. D = dentin.
Fig. 5. Relatively few nerve fibers are IR to SP and only occasionally approach the dentin (arrows).
Bars = 0.1 mm.







Figs. 6 and 7. Serial cross-sections from canine root pulp.
Fig. 6. Dense innervation of calcitonin gene-related peptide-immunoreactive (CGRP-IR) varicose fibers in the walls of blood vessels (V). Some free fibers are without any apparent connection to blood vessels (arrows).
Fig. 7. Same vessels (V) as in Fig. 6, rather sparsely supplied by nerves IR to SP. A few single SP-IR fibers (arrows) seem not to be associated with vessels.
Fig. 8. Some of the larger vessels (V) running centrally in the pulp showed thin, beaded NPY-IR fibers in the wall.
Bars = 0.025 mm.

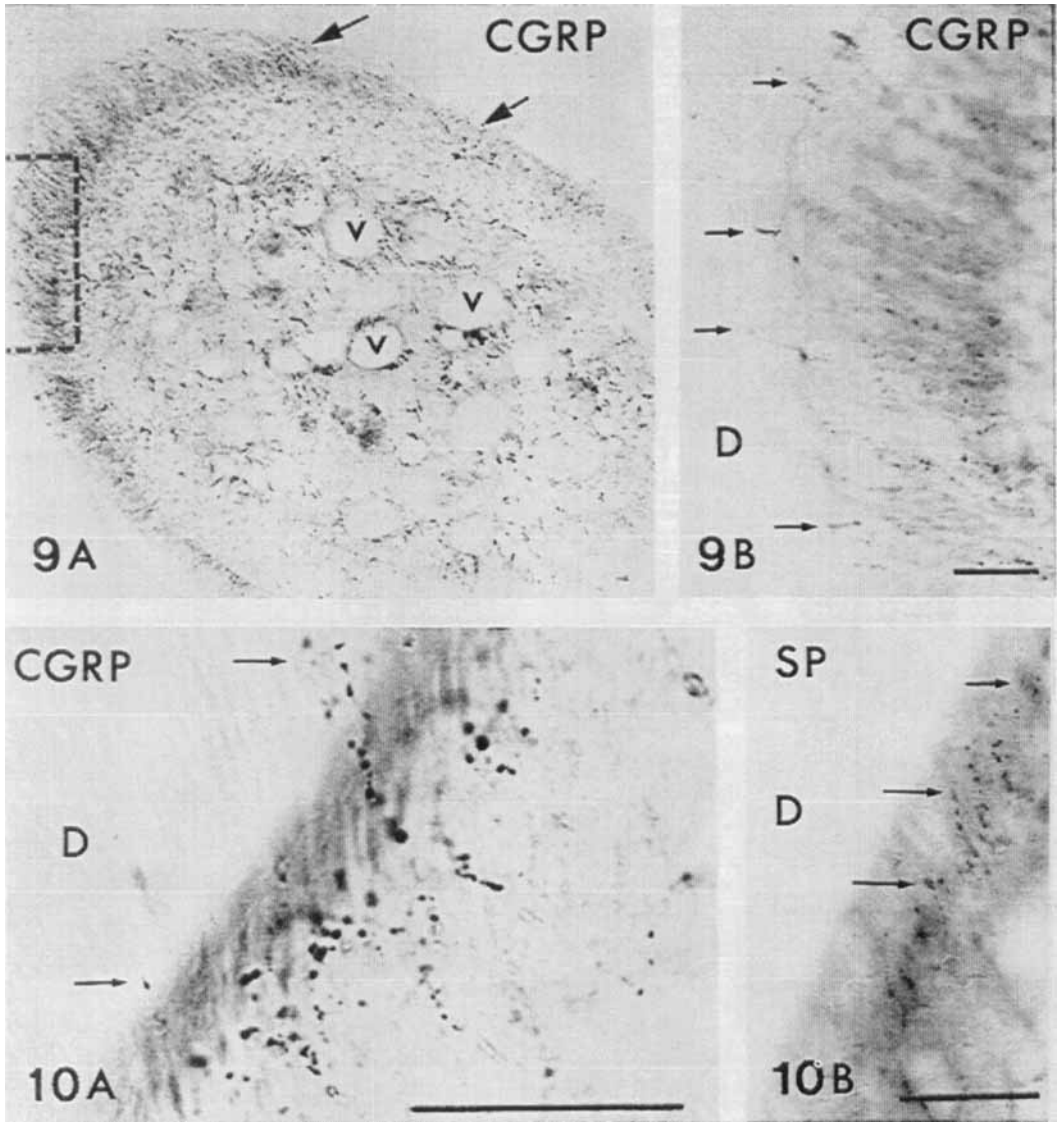
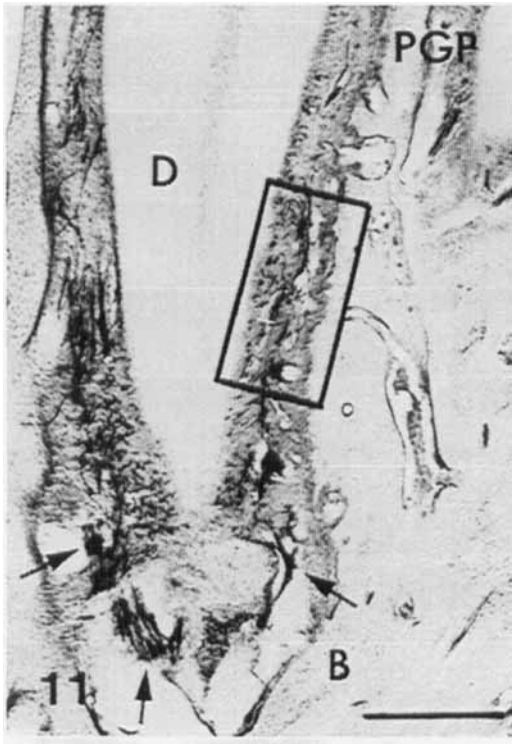
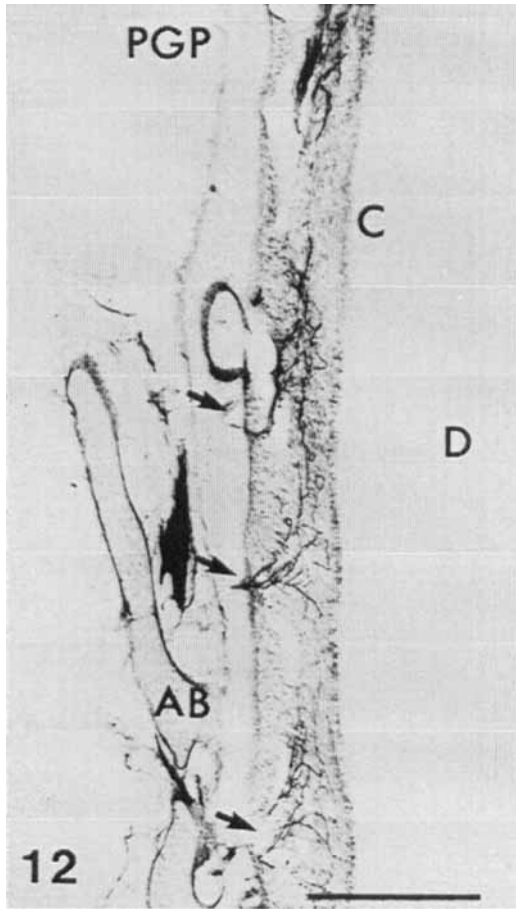


Fig. 9. Horizontal section of coronal canine pulp. 9A. Extensive innervation of calcitonin gene-related peptide-immunoreactive (CGRP-IR) fibers around blood vessels (V) and as a dense network in the subodontoblast/odontoblast layer (arrows). 9B. Outlined area from 9A. Numerous fibers are transversing the odontoblast layer and penetrating into the dentinal tubules (arrows). D = dentin.
Fig. 10. Horizontal serial sections of canine root pulp. 10A. CGRP-IR fibers in odontoblast area only occasionally extending into dentin (arrows). 10B. Relatively few substance P (SP)-IR fibers (arrows) in odontoblast layer in the root pulp, which did not penetrate into the dentin (D).
Bars = 0.05 mm.





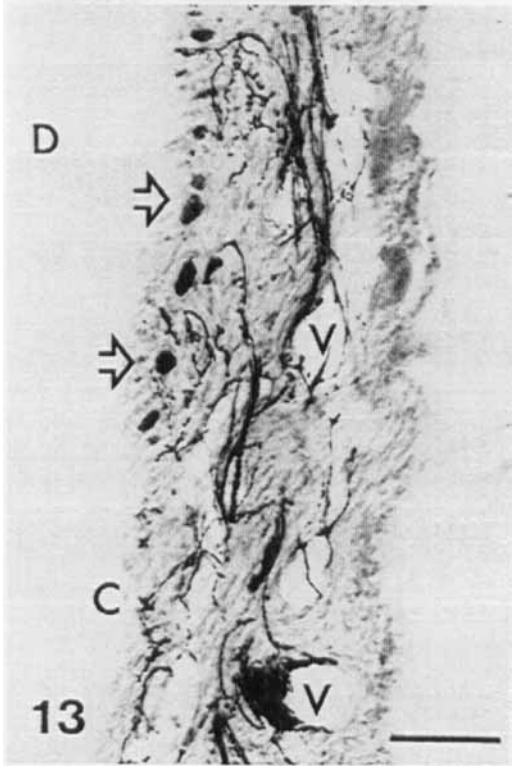


Fig. 11. Apical periodontal ligament (PDL) of incisor richly supplied with protein gene product (PGP) 9.5-immunoreactive (IR) nerves (arrows) from apical bone (B). D = dentin. Bar = 0.5 mm.

Fig. 12. Canine PDL, apical third. Numerous PGP 9.5-IR nerves (arrows) are approaching the PDL from lateral alveolar bone (AB). D = dentin; C =cementum. Bar = 0.5 mm.

Fig. 13. Enlarged area from Fig. 11. Numerous nerve fibers IR to PGP 9.5 are supplying the periodontal blood vessels (V). Extensive branching of nerve fibers adjacent to root surface covered with cementum (C). PGP 9.5-IR structures located in Malassez epithelium-like cells (open arrows). Bar = 0.1 mm.

Frozen sagittal and horizontal serial sections, 50 μm thick, were cut from the jaws on a freezing microtome. The immunoreactions for PGP 9.5, CGRP, SP, and NPY were evaluated on free floating sections as previously described (32). In brief, alternate serial sections from incisors and canines were incubated for 72 h with human PGP 9.5 polyclonal antibody (1:8000 dilution, UltraClone Limited, Cambridge, UK), or polyclonal antibody to rat CGRP (1:7000 dilution), or SP (1:5000 dilution) or NPY (1:4000 dilution, Cambridge Research Biochemicals, Cambridge, UK). Primary antibody binding sites were localized by means of the avidin–biotin method (ABC Kits, Vector Laboratories Inc., Burlingame, Calif., USA). Final visualization for all sections used diaminobenzidine (DAB, Sigma) plus 0.0125% H_2O_2.

Immunocontrols were routinely performed for all antibodies used. The sections were mounted on gelatin-coated slides and counterstained with Richardson's (1% methylene blue, 1% Azure II, 1% sodium borate, stock diluted 1:200) and analyzed in a Leitz photomicroscope.

Results

A considerably higher density of nerve fibers was observed in the dental pulp than in PDL and gingiva. The denser innervation in the pulp was observed for PGP 9.5-, CGRP- (Fig. 1), and SP-IR fibers.

Dental pulp

When compared with the complete pulpal innervation labeled with antiserum to PGP 9.5 (Fig. 2), most pulpal nerves were found to be CGRP-IR (Fig. 3), whereas considerably less fibers showed NPY- or SP-IR (Figs. 4 and 5). There was a denser innervation of CGRP-IR fibers in the coronal than in the apical pulp. Many fibers in the central pulp were associated with blood vessels, as a heavily stained network arrangement in their walls (Fig. 6). However, relatively thick fibers with varicosities showing CGRP-IR were also observed without any apparent connection to blood vessels (Fig. 6). Most fibers in the odontoblastic and subodontoblastic layer in the coronal pulp were immunoreactive to CGRP (Figs. 3 and 9), where they formed a dense network, which frequently penetrated into the dentin (Fig. 9B). Approximately three to four times more CGRP- than SP-IR fibers (Figs. 5 and 7) were consistently found in the pulp. The nerve fibers showing SP-IR were considerably fewer and thinner than the CGRP-IR fibers, as exemplified in the serial sections in Figs. 6 and 7. Most of the nerves IR to SP were localized to the vessel walls (Fig. 7), and only occasionally did they penetrate into the coronal dentin (Fig. 5). Compared with the coronal pulp, few CGRP-IR nerves entered the apical dentin (Fig. 10A), whereas SP-IR fibers only sporadically reached the odontoblast layer and predentin (Fig. 10B) in the apical pulp. The SP-IR nerves were not observed to enter root dentin (Fig. 10B).

NPY-IR nerves were mainly confined to the walls of the larger vessels running centrally both in coronal (Fig. 4) and apical (Fig. 8) pulp. The very thin, scarce, and beaded NPY-IR fibers were seldom found in the pulp periphery and were absent from the

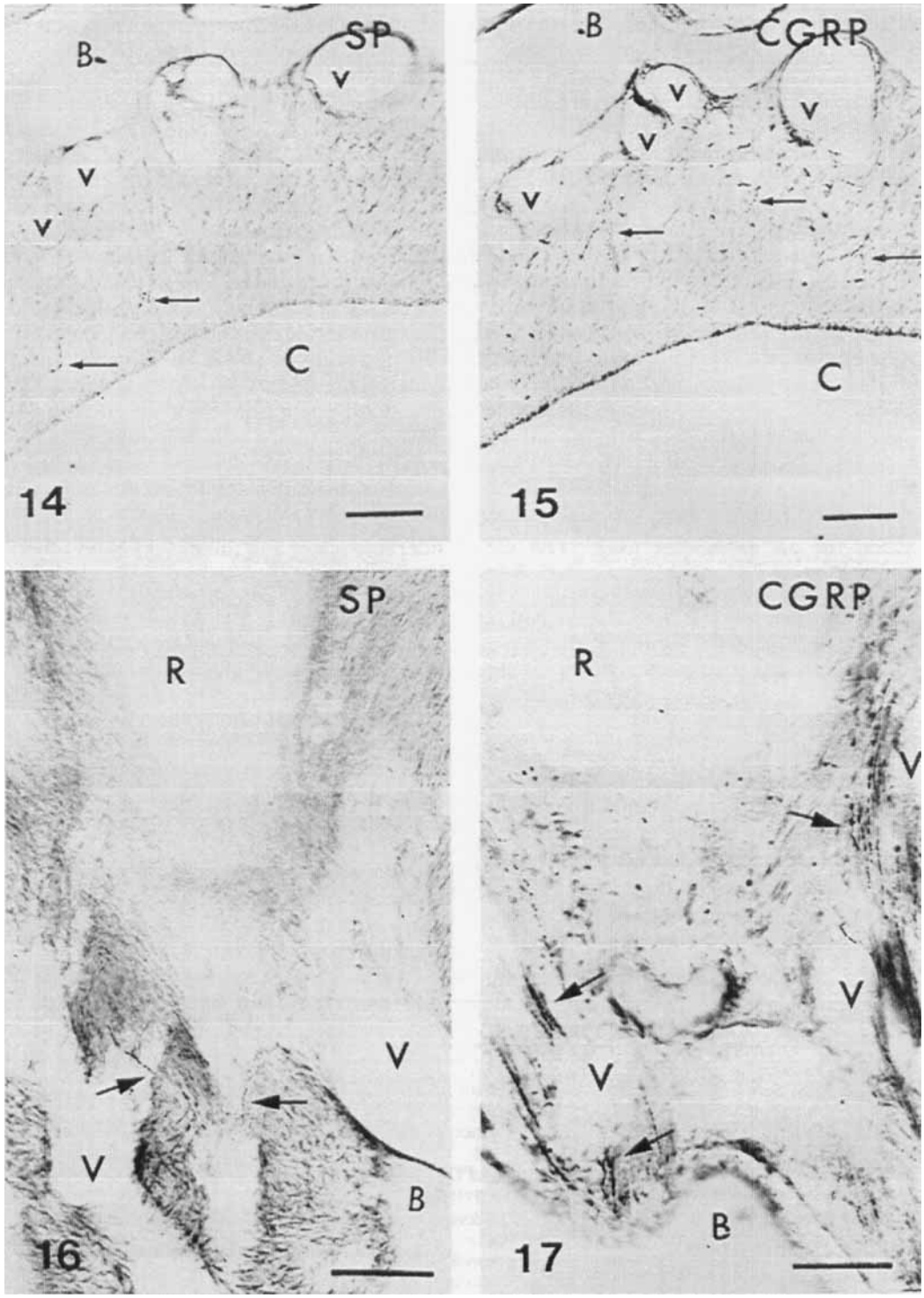
Overleaf

Figs. 14 and 15. Serial horizontal sections from canine periodontal ligament (PDL), midroot. Relatively few substance P-immunoreactive (SP-IR) fibers (Fig. 14) compared with calcitonin gene-related peptide (CGRP)-IR fibers (Fig. 15) were found. Both SP- and CGRP-IR nerves were located around blood vessels (V) and as free fibers (arrows). C = cementum.

Figs. 16 and 17. Sagittal serial sections of incisor apical PDL with SP- (Fig. 16, arrows) and CGRP-IR fibers (Fig. 17, arrows). B = alveolar bone; R = root; V = blood vessel. Bars = 0.1 mm.

Fig. 18. Neuropeptide Y-immunoreactive (NPY-IR) nerve fibers (arrows) along major blood vessels (V) in apical periodontal ligament (PDL) and alveolar bone (B). R = root.

Figs. 19 and 20. Apical third of cat canine showing mandibular canal (MC) and PDL. Large blood vessels (V) in the MC are shown at higher magnification in Fig. 20. A dense plexus of NPY-IR nerve fibers (arrows) was located in the walls of the vessels (V) in the MC, whereas the vessels in the PDL were rarely innervated by NPY-IR nerves. B = alveolar bone. Bars = 0.1 mm.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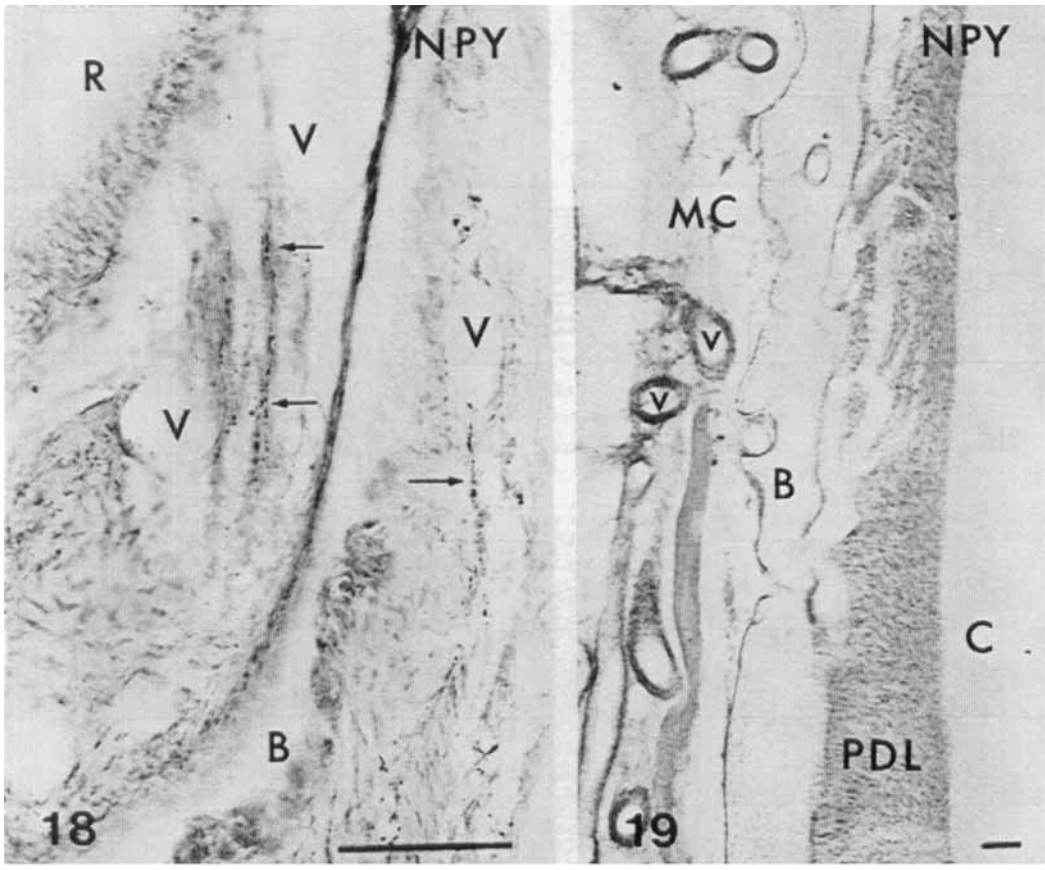
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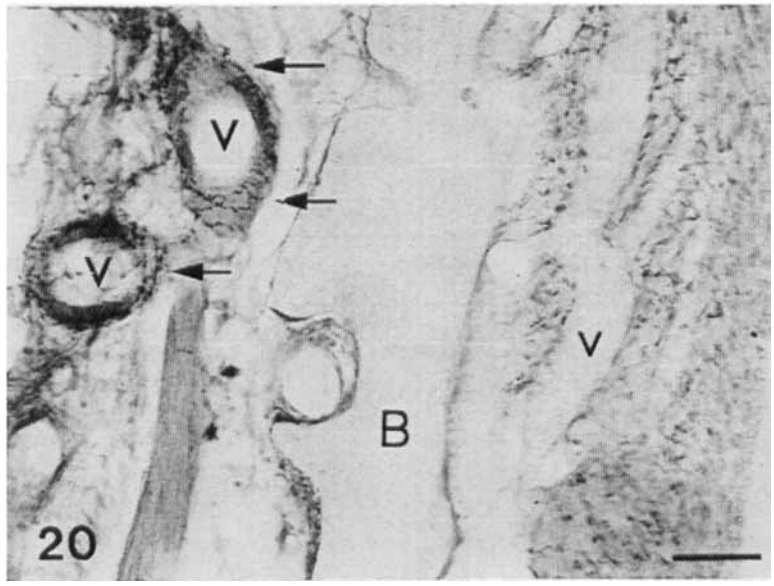
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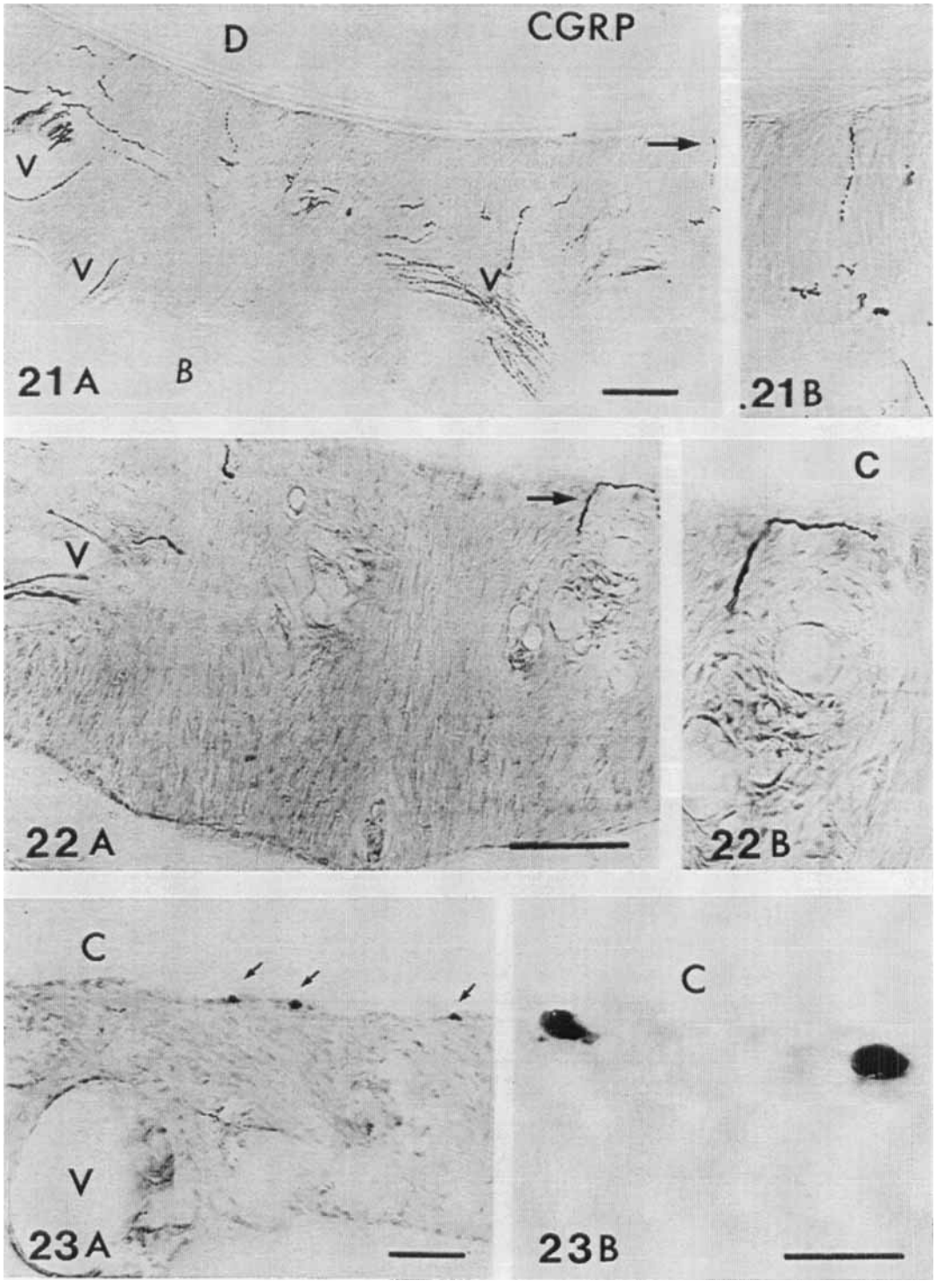
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subodontoblast/odontoblast layer and dentin (Fig. 4).

The periodontal ligament

Although less innervated than the dental pulp, the PDL was richly supplied with nerve fibers from apical (Fig. 11) and lateral (Fig. 12) alveolar bone, with extensive branching of nerves adjacent to cellular root cementum (Fig. 13), as demonstrated with antibody to PGP 9.5. Compared with the apical part, the midroot and cervical parts were more sparsely innervated. CGRP-IR fibers appeared more frequently than SP-IR fibers at all levels in the PDL (Figs. 14 to 17), whereas NPY-IR fibers were rarely found. Only occasionally could NPY-IR fibers be traced along large periapical blood vessels (Fig. 18). However, some of the larger blood vessels in the mandibular canal were densely innervated with NPY-IR nerves (Figs. 19 and 20), indicating that the NPY-IR fibers are mainly confined to relatively big arterioles or small arteries.

Both CGRP- and SP-IR nerves were mostly found in the walls of blood vessels (Figs. 14, 15, and 21A). However, CGRP-IR fibers extending close to the cementum, where no blood vessels are located, were relatively frequently observed (Figs. 21 and 22). Some of these CGRP-IR structures seemed to form round, coiled, nerve-like endings near the border between PDL and cementum (Fig. 23) or in parts of Malassez epithelium-like structures (Figs. 24A and 27). These structures also showed PGP 9.5-IR (Fig. 24B) but not SP- or NPY-IR.

Gingiva

In the gingiva PGP 9.5-IR nerve fibers were found extending between the epithelial cells up to the surface (Fig. 25). Some of these fibers were CGRP-IR as well (Fig. 26). In some areas of the basal layer of the gingival epithelium, round cell-like structures in the rete pegs were PGP 9.5-IR (Fig. 25) or CGRP-IR (Figs. 26–28).

No SP- or NPY-IR fibers were observed among the gingival epithelial cells. However, in the submucosa SP-, NPY-, and CGRP-IR (Fig. 26) nerve fibers were found.

Discussion

The cat pulp displayed significantly more CGRP-IR nerves than both the PDL and gingiva, indicating that CGRP fibers are of particular importance for the pulp function. The present study in cat incisors and canines shows that the vast majority of pulpal nerves contained the neuropeptide CGRP, which contrasts with the finding of Luthman et al. (13) that the major part of nerve fibers in the human pulp is non-peptidergic but is in good agreement with earlier findings in rat molars (3, 14, 19, 20). In the present study a substantially greater part of the nerves both in pulp and PDL was IR to CGRP than to SP, approximately three to four times more, corresponding fairly well with that observed by Fried et al. (3).

Contrary to the occurrence of NPY and SP, a remarkable abundance of CGRP-IR nerves was found in no apparent proximity to blood vessels, as in the odontoblast/dentin area and adjacent to root cementum, suggesting that this neuropeptide has functions in addition to blood flow regulation (33). As an osteogenic stimulating effect of CGRP on bone colonies has been observed in vitro (34), this might denote that the CGRP-IR structures located adjacent to cementum and in close relationship to the odontoblast processes might possess a stimulating effect on hard tissue.

The function of CGRP in fibers within dentinal tubules is still obscure. A main find-

Figs. 21 to 23. Horizontal sections from the middle third of canine periodontal ligament (PDL) showing calcitonin gene-related peptide-immunoreactive (CGRP-IR) nerves. Most CGRP-IR fibers were located around blood vessels (V, Figs. 21A, 22A, and 23A; bars = 0.1 mm). However, fibers extending adjacent to cementum (enlarged areas, Figs. 21B and 22B) were frequently observed. Some CGRP-IR structures were located on the border between PDL and cellular cementum (arrows, Fig. 23A). Fig. 23B. Higher magnification of CGRP-IR structures adjacent to cementum (C). D =dentin. Bar = 0.05 mm.

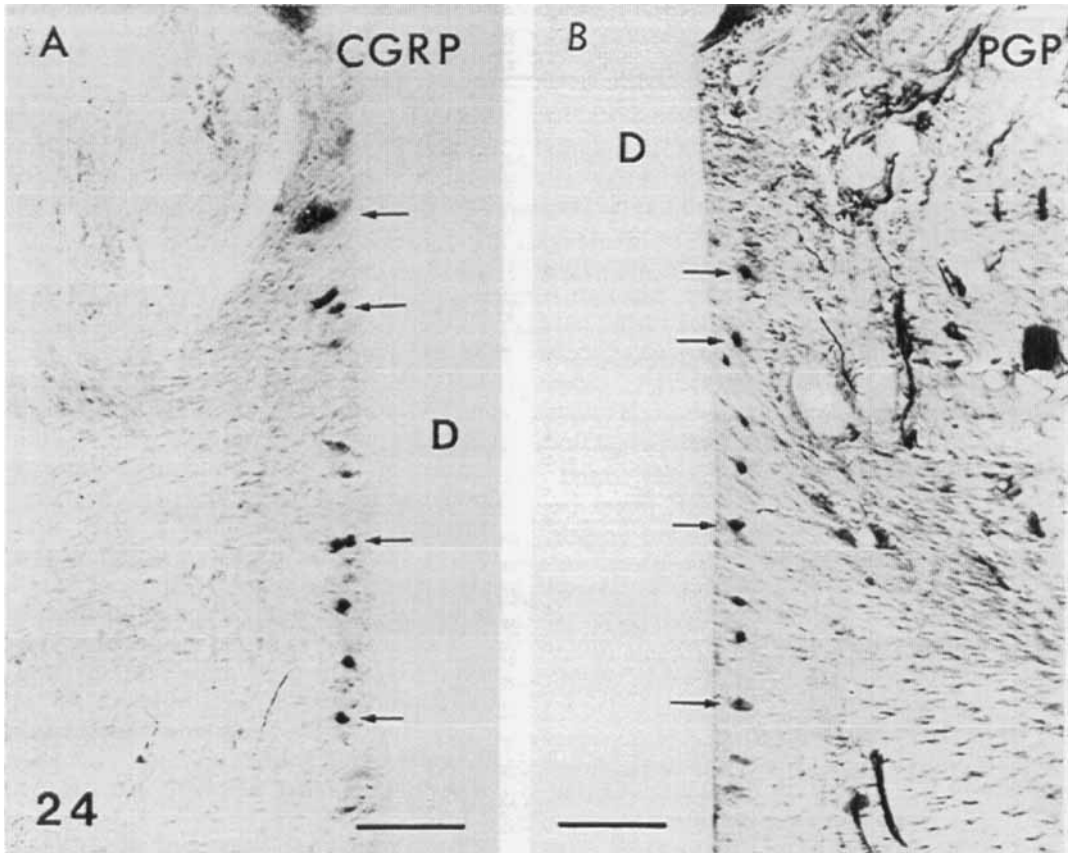


Fig. 24. Longitudinal serial sections from cervical periodontal ligament of canine, showing accumulation of immunoreactivity for calcitonin gene-related peptide (CGRP) (A) and protein gene product (PGP) 9.5 (B) in cell-like structures (arrows) in Mallassez epithelium. Bars = 0.1 mm. D = dentin.

ing in the present study was that most nerve fibers in predentin/dentin displayed CGRP-IR, which might suggest that in addition to afferent nociceptive impulse conduction, these fibers might simultaneously, in efferent direction, release the vasodilator CGRP and increase pulpal blood flow. While the previous general view has been that the unmyelinated C-fibers are responsible for a vascular defense reaction in the pulp in response to clinical procedures such as cavity preparation (26), the present study supports the findings of Vongsavan & Matthews (16) that CGRP-IR A fibers in dentin may be responsible for this reaction. Recent findings that drilling partway into dentin produces depletion of CGRP (35) also favors this view.

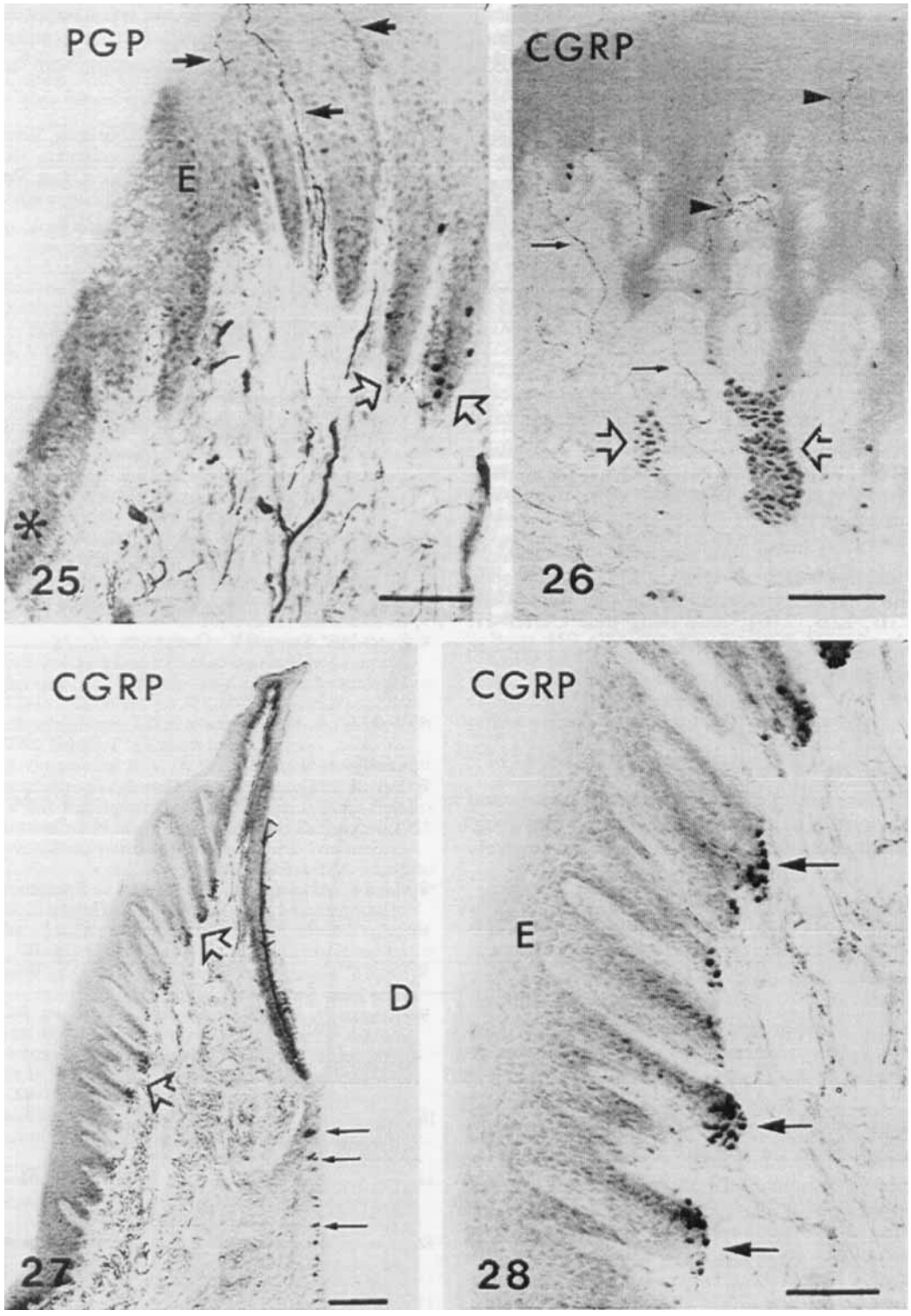
In contrast to previous findings in cat pulp

Fig. 25. Sagittal section of canine gingiva, showing numerous protein gene product (PGP) 9.5-immunoreactive (IR) nerves, with some fibers (arrows) running towards epithelial (E) surface. Some cells (open arrows) in the basal layer of the epithelium showed PGP 9.5-IR. Junctional epithelium (*).

Fig. 26. Horizontal section of canine gingiva, showing calcitonin gene-related peptide (CGRP)-IR nerves in epithelium (arrowheads), submucosa (arrows), and round cell-like structures in the rete pegs (open arrows).

Fig. 27. Sagittal section of canine gingiva, showing CGRP-IR in Malassez epithelium cell-like structures (arrows) and in rete pegs of gingival epithelium (open arrows). D = dentin. The CGRP-IR round cell-like structures in the basal layer of epithelium (E) are shown at higher magnification in Fig. 28 (arrows).

Bars =0.1 mm.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(36, 37), the SP-IR fibers only occasionally penetrated into the dentinal tubules. Thus, the present distributions of SP- and CGRP-IR fibers were *not* very similar and disagree with findings with double immunostaining for CGRP and SP showing that *all* CGRP-containing nerve fibers were also SP-IR in cat pulp (36). However, some of this discrepancy might be due to age changes (8).

In the central pulp most nerves IR to CGRP, SP, and NPY were localized to the vessels walls, clearly suggesting their participation in regulation of blood flow and/or vascular permeability.

In the PDL extensive branching of nerves adjacent to cellular root cementum was consistently observed (Fig. 13), whereas mid-root and cervical areas of the PDL were more sparsely innervated. SP-IR fibers were found less frequently than CGRP fibers at all levels of the PDL (Figs. 14 to 17). The SP fibers never extended to the vicinity of the non-vascular cementum, as was frequently the case with CGRP-IR fibers (Figs. 21B, 22B, 23B), indicating that CGRP-IR nerves may have distinct functions in addition to blood flow regulation. The present distribution of CGRP-IR fibers clearly matches that reported earlier using axonal transport or immunotechniques (2, 4, 38).

The vessels in the mandibular canal showed a dense NPY-IR innervation (Fig. 20), whereas NPY-IR nerves were rarely found in the PDL, except along the larger periapical blood vessels (Fig. 18). In the dental pulp and gingiva, too, the NPY-IR nerve fibers were mostly observed in connection with small arteries or relatively big arterioles. This is in accordance with previous findings (6) and might suggest that sympathetic innervation is mainly located to relatively big vessels.

An unexpected but consistent finding was that PGP 9.5 and CGRP-IR seemed to accumulate in round cell-like structures closely connected to, or part of, the Malassez epithelium both in the apical and cervical PDL (Figs. 13, 23, 24, 27). Round cell-like structures localized in the base of the epithelial rete pegs also showed PGP 9.5 and CGRP-IR (Figs. 25, 26, 28). For exact identification of these IR cell-like structures both in gingiva and PDL, further studies using immunoelectron microscopy will be necessary.

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