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Neurochemistry of the Gustatory Subgemmal Plexus

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Abstract

Nerve fibers present in the basal plexus of the vallate papilla of the rat tongue were analyzed using cytochemical, immunocytochemical and ultrastructural methods to investigate whether the subgemmal plexus is subdivided into neurochemical compartments and to provide a clear definition of the reciprocal spatial relationships between nitrergic, peptidergic and acetylesterase positive structures. Several neuronal fibers were detected under the chemoreceptorial epithelium. Some of these fibers were in contact with the taste buds and in some cases neuronal projections were also present between the buds or inside them; some others fibers were present below this layer but in a more peripheral area. Antibodies against CGRP, SP and CCK stained fibers just below the chemoreceptorial epithelium, whereas fibers more distally located were immunolabeled by anti VIP, NOS-1 and NF-200 antibodies. Some double staining experiments were conducted using confocal microscopy. Other sections were processed cytochemically for AChE and subsequently for NADPH-d in colocalization experiments. All the data obtained using these techniques confirmed the results obtained with single immunostaining, as did the ultrastructural results. In conclusion, the present work demonstrates that the subgemmal plexus is a bilayered structure, suggesting that the complex relationship between the two layers plays a pivotal role in taste and in the control of processes ancillary to taste, such as control of vascular or secretory mechanisms.

Key words: confocal microscope, gustatory system, immunohistochemistry, neuropeptides, tongue

Introduction

The vallate papilla (VP) of the mammalian tongue is an important chemoreceptor organ rich in taste buds, which is mainly involved in control of food intake. The gustatory epithelium covering this structure has been extensively studied, but less attention has been paid to the neurochemistry of the gustatory basal (subgemmal) plexus (Miyawaki *et al.*, 1996; Daikoku *et al.*, 1999; El-Sharaby *et al.*, 2001). In this specific area, various nerve components are in contact with one another, establishing complex interrelations (Sbarbati *et al.*, 2002). Light microscopy evaluations have shown that both intragemmal and extragemmal fibers converge in the subgemmal plexus (Sbarbati *et al.*, 2002). Some of these fibers have a sensory function, while others probably operate in a regulatory pathway. In addition, the intrinsic neurons' nerve processes reach the basal plexus. Intrinsic neurons belong to an intrinsic nervous system (INS) which plays a pivotal role in the regulation of the VP and of the annexed serous von Ebner gland (VEG; Sbarbati *et al.*, 2001). Previous studies have demonstrated the remarkable complexity of this INS; it has been hypothesized that it shares chemical and structural characteristics with the enteric nervous system and could probably be considered a specialized portion of the latter (Kirchgessner and Gershon, 1990; Goyal and Hirano, 1996; Sbarbati *et al.*, 2000). Ultrastructural and cytochemical examination has pointed out that the neurons of the INS are nitrergic elements, while afferent nerve fibers are mainly composed of peptidergic elements (Sbarbati *et al.*, 2002). Both the behavior of the nitrergic fibers in the basal plexus and their spatial relationships with peptidergic components are still unknown. In spite of such complexity and importance, there are no specific studies in the literature concerning the neurochemistry of the subgemmal plexus. Of particular interest are the results obtained in humans by McDaniel (1999), who described the basal (subgemmal) nerve plexus as a tortuous neural proliferation associated with the taste buds, with ganglion cells and intergemmal/intragemmal branches.

In the present study, using cytochemical, immunohistochemical and ultrastructural immunocytochemical techniques, we found new data on this system and we tried to carry out a detailed study of the basal plexus. Some double staining experiments were conducted using confocal

microscopy. The aim of this investigation was to analyze whether the basal plexus is subdivided into neurochemical compartments and to provide a clear definition of the reciprocal spatial relationships between nitrergic and peptidergic structures.

Materials and methods

Wistar rats of both sexes weighing 150–200 g were obtained from the Morini Company (Reggio Emilia, Italy), kept at the departmental animal facility and fed a standard laboratory diet *ad libitum* (D.L. no. 116/92–27/01/92).

Immunohistochemistry

Animals were anesthetized with ether and handled in accordance with the guidelines for animal experimentation laid down in Italian law.

The animals were perfused intra-aortically with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). For VAChT detection, 0.4% picric acid was added to the fixative. The lingual tissue was post-fixed by immersion for 1 h in the same fixative. Afterwards, the tissue was transferred into a 10% sucrose solution in phosphate buffer. Blocks of lingual tissue containing the vallate papillae as well as adjacent epithelium, muscles and glands were dissected. Tissue blocks were sectioned on a freezing microtome. Parallel free-floating sections (30–40 μm) were collected in phosphate buffer saline (PBS, pH 7.4) and washed in several changes of this solution before further processing. In other rats, the tongues were removed after death and fixed by immersion in 4% neutral buffered formalin from 2 to 6 h at 4°C, rapidly dehydrated using alcohol steps, transferred to xylol and embedded in paraffin (melting point 52°C; Merck, Darmstadt, Germany). Paraffin sections (10–12 μm) were cut and stretched at 45°C, allowed to dry and stored at 4°C until use.

For the immunohistochemical experiments free-floating sections were processed without pre-treatment, while paraffin sections were deparaffinized in xylol and dehydrated in a graded series of ethyl alcohol. Free-floating as well as paraffin sections were incubated for 10 min with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidases. Sections were then incubated for 15–20 min with 3% normal swine serum diluted in PBS or 3% bovine serum albumin (BSA) diluted in PBS. Afterwards the sections were incubated with the following primary antibodies: (i) rabbit anti-calcitonin gene related peptide (CGRP; kindly provided by Dr Sternini of UCLA), dilution 1:4000, incubated overnight at 4°C; (ii) rabbit anti-nitric oxide synthase type-1 (NOS-1; Boehringer, Mannheim, Germany), dilution 1:50, incubated overnight at 4°C; (iii) goat anti-vesicular acetylcholine transporter (VAChT; Chemicon, Temecula, CA), dilution 1:2000, incubated overnight at 4°C; (iv) mouse monoclonal antibody specific for neurofilament 200 kDa (NF 200, clone RT97; Novocastra Laboratories Ltd, DBA Italy), dilution 1:1000, incubated overnight at 4°C; (v) rabbit polyclonal anti-substance P (SP; Zymed Laboratories, South San Francisco, CA), used undiluted, incubated for 1 h at room temperature; and (vi) rabbit anti-cholecystokinin-8 (CCK-8; Oncogene), dilution 1:1000, incubated overnight at 4°C. Sections were washed in PBS and then incubated with a secondary antibody (Dako, Glostrup, Denmark). An avidin–biotin complex (ABC) technique was used to reveal sites of antigen–antibody reaction. For the ABC method a commercial kit (ABComplex/HRP, Code No. K0355; Dako) was used. Kit instructions were followed with regard to dilution and incubation times. Peroxidase activity was revealed by diaminobenzidine (Sigma, St Louis, MO). Then the sections were dehydrated through ethanols, cleared in xylene and coverslipped with entellan. In parallel with the above immunohistochemical procedures, controls were conducted replacing the primary antibody with 10% non-immune serum or with PBS–BSA 3%. Further controls were conducted omitting the secondary antibody.

Immunofluorescence

Frozen sections on slides were washed in PBS at room temperature and permeabilized for 1 h in PBS containing 0.5% Triton X-100 and 3% BSA or 10% normal serum; the same solution was used to dilute the antibodies. Subsequently, some sections were incubated with NF 200 used at dilution 1:1000 overnight at 4°C; other sections were incubated with CGRP used at dilution 1:4000 overnight at 4°C. Slides were washed twice in PBS and then incubated for 1 h at room temperature with a secondary fluorescein (FITC) conjugated rat anti-mouse IgG antibody (Jackson Laboratories Inc., Baltimore, PA) or with a rhodamine (TRITC) conjugated goat anti-rabbit IgG (Jackson Laboratories); both the secondary antibodies were used at dilution 1:150. After two washes in PBS the sections were mounted under coverslips with *N*-propyl gallate, prepared according to Giloh and Sedar (1982).

In some experiments, double labeling for NF-200 and CGRP was performed. After a first labeling with anti NF-200 as above, sections were washed three times with buffer and incubated with the CGRP antibody, then washed and reacted with the second antibody for 60 min at room temperature. Sections were finally rinsed and mounted in *N*-propyl gallate. Sections treated as above, but in the absence of the primary or secondary antibody, were used as controls. Sections were studied using a Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and helium/neon (543 nm) excitation beams.

NADPH-d cytochemistry

Animals ($n = 6$) were deeply anesthetized with ether and then perfused, using a steel cannula inserted through the heart into the ascending aorta, with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Blocks of lingual tissue containing the VP as well as adjacent epithelium, muscles and glands were rapidly dissected. Tissue blocks were post-

fixed in a solution of 4% paraformaldehyde for 4 h and then seeded overnight in a solution of 10% glucose. Tissue blocks were frozen sectioned (30–40 μm) and mounted on gelatin-coated slides. The sections were then washed in PBS, pre-incubated in PBS with 0.2% Triton X-100 in a shaker at room temperature (5 min) and then incubated in a solution containing 1% NADPH (reduced form; Sigma), 0.5% nitroblue tetrazolium (Sigma) and 0.3% Triton X-100 in PBS at 37°C for 2 h. The sections were washed in PBS, dehydrated in graded alcohol, cleared in xylol, coverslipped with Entellan and studied under the microscope with bright-field illumination.

For ultrastructural examination, the specimens of the dorsal surface of the tongue ($n = 8$), each including the single VP, were removed, fixed by immersion in glutaraldehyde 2% in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in the same buffer for 1 h and dehydrated in graded ethanols. The specimens were embedded in Epon–Araldite and sectioned in an Ultracut E (Reichert) ultramicrotome. The semithin sections were stained with toluidine blue, while ultra-thin sections were stained with lead citrate and uranyl acetate and observed under an EM 10 electron microscope (Zeiss).

AChE cytochemistry for light microscopy

Enzymatic staining of AChE for light microscopy was performed following the direct thiocholine method of Karnowsky and Roots (1964). Six adult Wistar rats were used for this study. Tongues were removed from animals anesthetized with diethyl ether and the VP was immediately excised and fixed in 4% paraformaldehyde in 0.4 M phosphate buffer for 1 h at room temperature. After fixation, tissues were put into 20% sucrose in phosphate buffer for 1 h at 4°C and sectioned on a freezing microtome (Reichert-Jung). Free-floating sections (thickness, 30 μm) were placed into polystyrene culture plates at room temperature. The sections were preincubated with 0.1 M acetate buffer, pH 6 for 30 min and incubated for 30 min in AChE staining solution containing: 5 mg of acetylthiocholine iodide; 0.5 ml of 0.1 M sodium citrate; 1 ml of 30 mM copper sulfate; 1 ml of 5 mM potassium ferricyanide; 1 ml of distilled H_2O; and 6.5 ml of 0.1 M sodium acetate buffer, pH 6.0. Control sections were incubated without acetylthiocoline iodide. After several rinses in acetate buffer, free-floating sections were mounted on glass slides, dehydrated and coverslipped with Entellan. Sections were examined using an Orthoplan 2 photomicroscope (Leitz).

AChE cytochemistry for electron microscopy

Enzymatic staining of AChE for electron microscopy was performed according to the copper–glycine method of Topilko and Caillou (1985). Seven adult Wistar rats were used for this study. Tongues were removed from animals anesthetized with diethyl ether and the VP was immediately excised, fixed in 1.44% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8, with 0.042 M NaCl and 0.0021 M acetylcholine chloride for 1 h at room temperature and transferred into 0.1 M cacodylate buffer with 0.042 M NaCl at 4°C for 4 h. After washing in 0.1 M sodium acetate, pH 5.2 for 10 min, the specimens were incubated for 16 h at 4°C in medium containing: 0.53 ml copper sulfate, 0.1 M; 5 mg glycine; 0. 2 ml sodium acetate, 1 M; 0.4 ml distilled water; 2.4 ml sodium sulfate, 0.085 M; and 3.5 mg acetylcholine iodide (or without acetylcholine iodide for controls). The specimens were rinsed in sodium acetate buffer for 1 h, treated with 0.25 % ammonium sulfide in saline for 15 min and then washed in 0.1 M cacodylate buffer (pH 6.8). After postfixation in 1% OsO_4 in the same buffer, the tissues were dehydrated in graded concentrations of acetone and embedded in a mixture of Epon and Araldite. Ultrathin sections were observed unstained in an EM 10 electron microscope (Zeiss).

Co-localization of AChE with NADPH-d

In co-localization experiments, the method involved two further steps of staining. In a first step we performed AChE staining on floating slices, using the methods described above. The sections were subsequently mounted in glycerol and examined with a light microscope (Leitz). Images were recorded using a KY-F58 CCD camera (JVC). In a second step, the same slices used for AChE detection were washed in PB and processed for NADPH-d cytochemistry. For light microscopy, the stained sections were mounted with Entellan. Color plates for evaluating the co-localization pattern on the same areas were made using Image Pro Plus software.

Results

Immunostaining of the subgemmal plexus and spatial distribution of nerve fibers

The neuronal fibers present in the subgemmal plexus were analyzed immunohistochemically using a panel of neuronal antibodies.

A large number of neuronal fibers were detected under the chemoreceptorial epithelium (Figures 1a–f, 2a–f and 4a–d). Some of these fibers were in contact with the taste buds and in some cases neuronal projections were also present between the buds or inside them (Figures 1c and 2d,f). A similar number of neuronal fibers was present below this layer but in a more peripheral area. Some of these fibers were also distributed around the vessels and towards the serous glands (Figure 3e,f).

Antibodies against CGRP, SP and CCK stained fibers running in direct contact with chemoreceptorial epithelium (Figure 1a–c), whereas more distally located fibers were immunolabeled by anti VIP, NOS-1 and NF-200 antibodies. A thin layer of connective tissue was regularly present between these stained fibers and taste buds (Figure 1d–f).

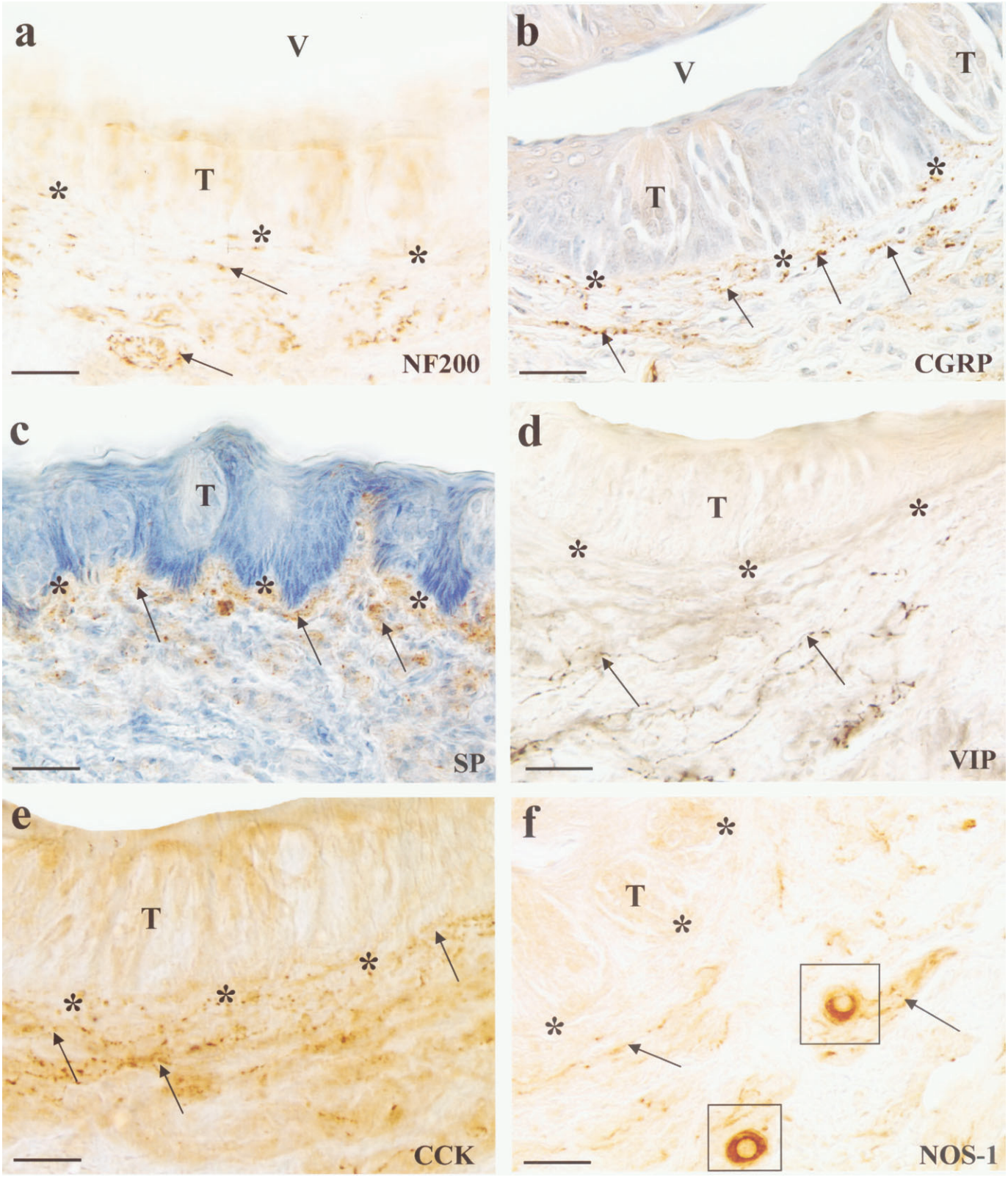

Figure 1 Immunolabeling of chemoreceptorial epithelium of rat circumvallate papillae for different neuropeptides: **(a)** NF-200, **(b)** CGRP, **(c)** SP, **(d)** VIP, **(e)** CCK, **(f)** NOS-1. In (a, d, f) deeply located fibers (arrows) are immunolabeled by anti NF-200, VIP and NOS-1 antibodies; a thin layer of connective tissue is regularly present between stained fibers and taste buds. In (b, c, e) note the network of fibers (arrows) running in direct contact with the region in which taste buds (T) are present. These fibers are clearly stained by antibodies against CGRP, SP and CCK. In panel (f), NOS-1 positive ganglion cells are surrounded by squares; V, vallus around the VP; the basal lamina is marked by asterisks. Scale bars = 30 mm.

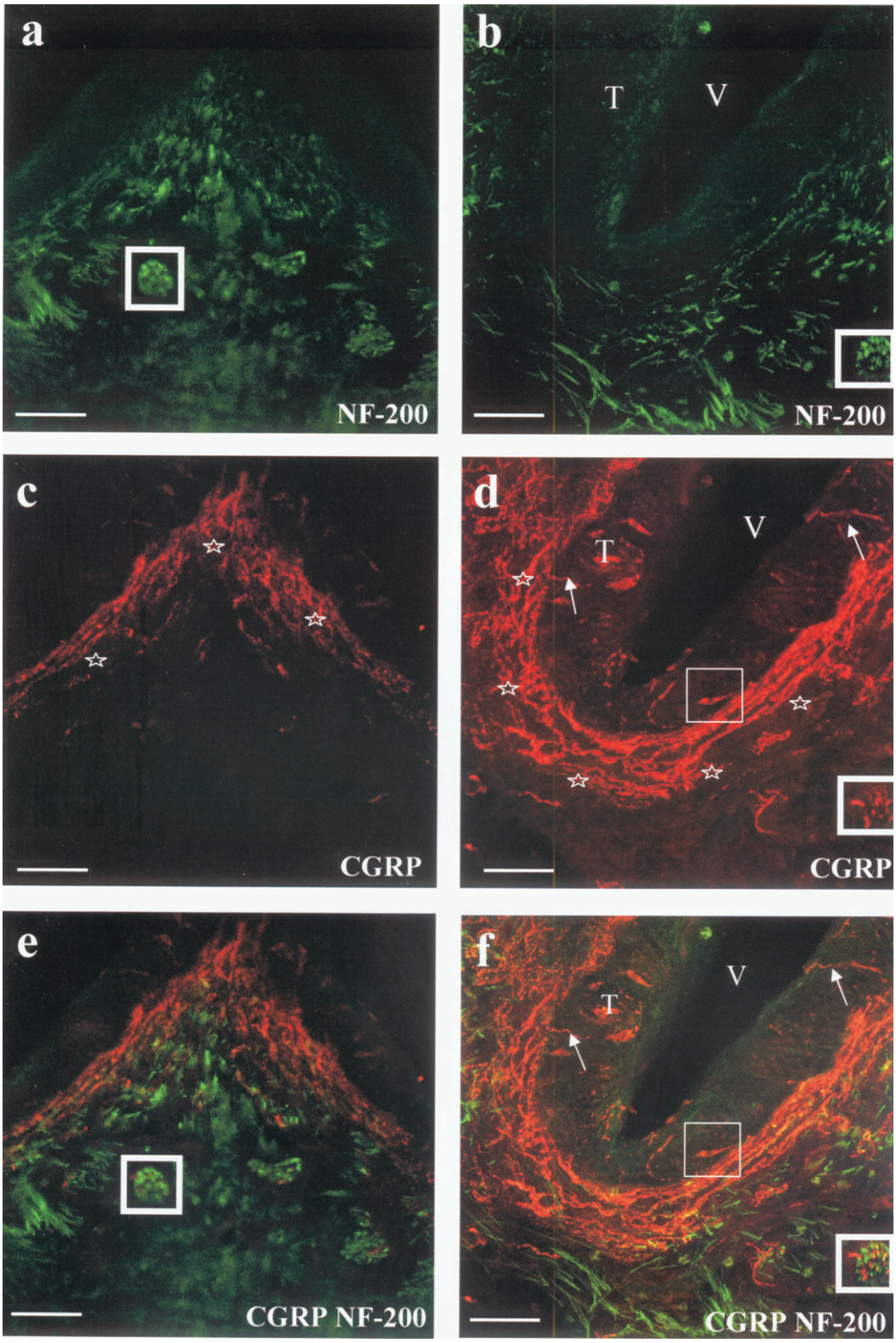

Figure 2 Confocal images that show the relationships between NF-200-IR and CGRP-IR nerve fibers. **(a, b)** Network of nerve fibers positive for NF-200 but not CGRP-IR. It can be seen that the positive fibers are parallel to the chemoreceptorial epithelium, but not in direct contact with it. **(c, d)** CGRP-IR (stars) is present in a group of nerve fibers located below the gustatory epithelium. **(e, f)** The same optical sections, differentially illuminated to reveal the localization of NF-200-IR and CGRP-IR nerve fibers. NF-200-IR fibers are clearly visible running under the CGRP-IR fibers. Intraepithelial CGRP positive fibers (arrows) are visible in (d, f); intragemmal taste cells are surrounded by square; trunks of nerve fibers are surrounded by heavy square; V, vallus around the VP. Scale bars = 70 mm (a, c, e); 43 mm (b, d, f).

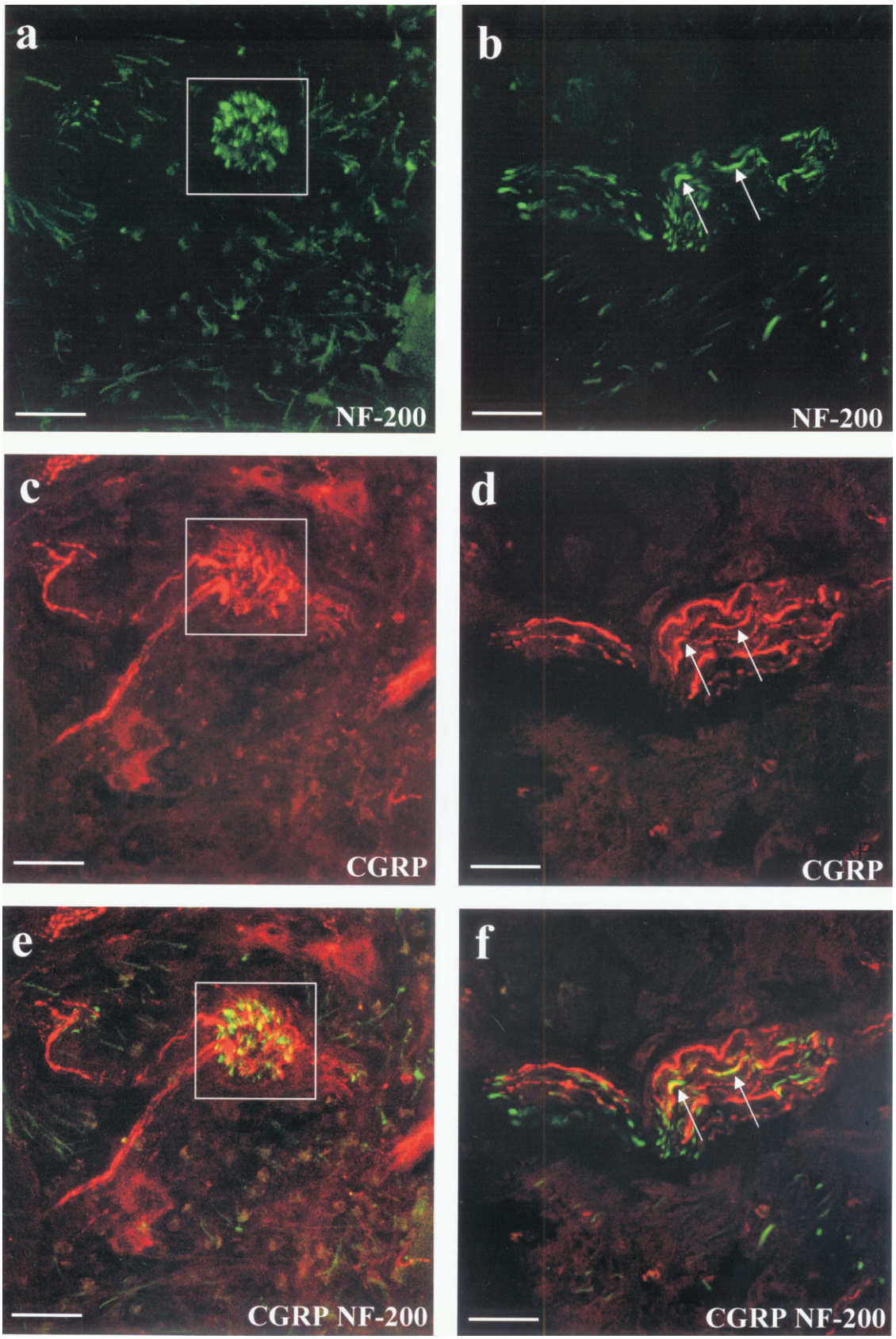

Figure 3 Confocal images of NF-200-IR and CGRP-IR in nerve trunks (square) located in the connective tissue of the papilla. In these nerves, NF-200-IR and CGRP-IR fibers (arrows) are not spatially segregated. **(a, c, e)** Transverse sections, scale bars = 28 μm **(b, d, f)**. Longitudinal sections, scale bars = 18 μm.

Immunostaining for CCK (Figure 1c) brought to light some interesting data: the pattern of fibers positive for this antibody involved a wider band of reactivity in comparison to other markers used, even if the density of immunostained fibers appeared similar to SP (Figure 1b) and CGRP (Figure 1a).

CGRP-IR fibers were visible in the perigemmal epithelium between the buds (Figure 2d,f).

Colocalization for NF-200 and CGRP

Confocal microscope examination of colocalization experiments confirmed that below the gustatory epithelium, immunoreactivity for NF-200 and CGRP was present in different groups of nerve fibers: NF-200-IR fibers were localized under the CGRP positive fibers (Figures 2e,f and 3e,f). A different pattern of distribution of NF 200-IR and CGRP-IR fibers was also found in nerve trunks directed towards the VEG and around the vessels (Figure 3e,f).

Colocalization experiments using AChE and NADPH-d

Some specimens were treated for colocalization experiments, by staining with AChE and then NADPH-d (Figure 4a–d). Using this approach we also found that two layers exist in the basal plexus. The connective layer directly in contact with taste buds was characterized by intense staining with AChE. The enzyme AChE was not able to label nerve

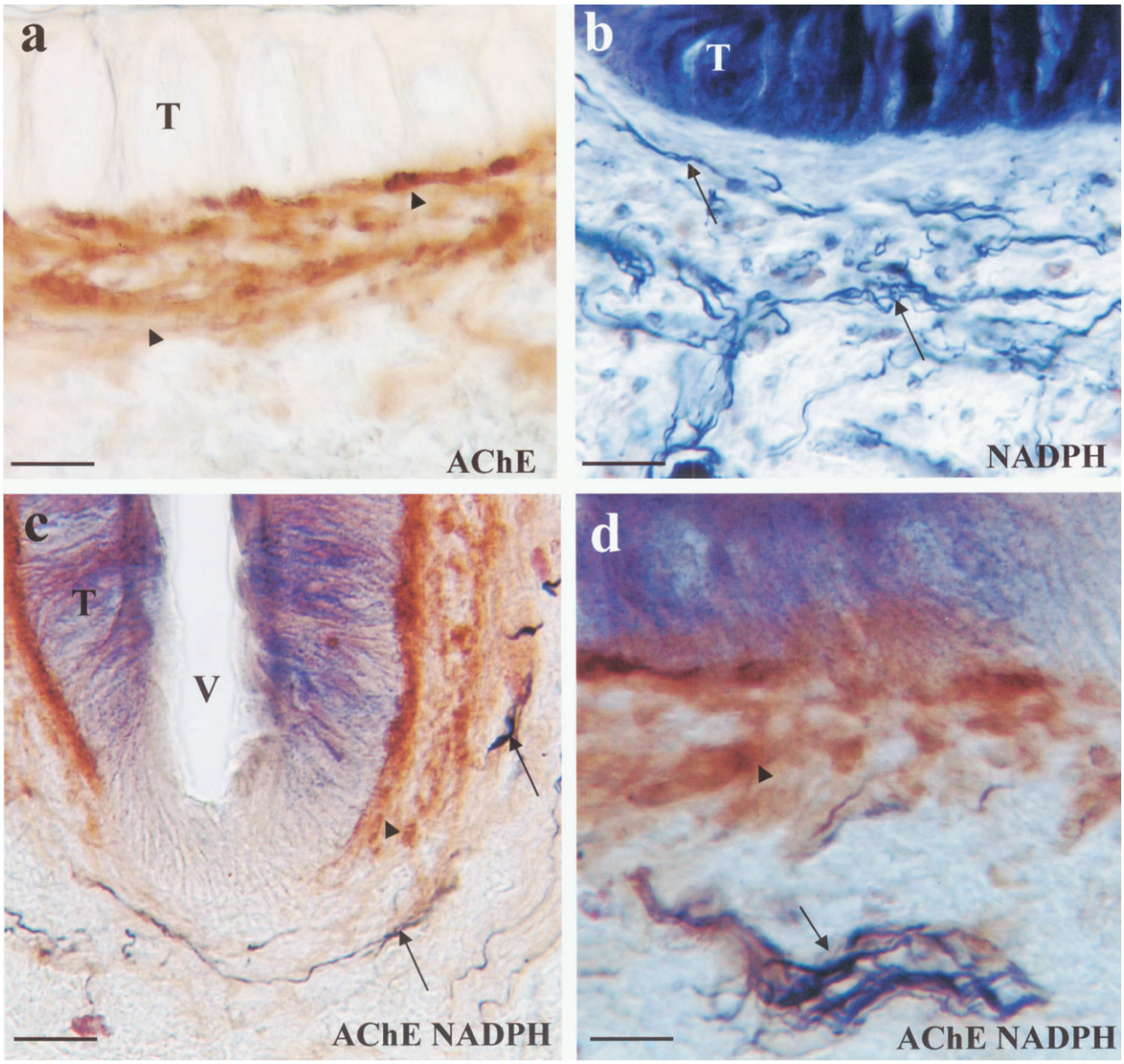

Figure 4 Colocalization of AChE and NADPH-d positive structures. **(a)** Enzymatic staining for AChE: the connective layer just below the taste buds shows an intense immunoreactivity for AChE marker (arrowheads). **(b)** Nitrergic fibers cytochemically stained by NADPH-d (arrow) are present in the connective tissue but not in contact with the chemoreceptorial epithelium. **(c, d)** In the same section it is possible to observe double staining for AChE and NADPH-d; note that the nitrergic fibers run parallel to the basal lamina of the epithelium below the taste buds. Scale bars = 30 μm (a, b); 35 μm (c); 20 μm (d).

fibers, but the reaction product of this staining was fully present at the extracellular level, as we demonstrated by using TEM techniques in the same tissues. Nitrergic fibers were found below this acetylesterase structure and they ran parallel to the basal lamina of the epithelium. Their distribution was fully consistent with NF-200 and with NOS-1 (Figure 1f) nerve fiber staining.

Ultrastructural cytochemistry of acetylcholinesterase area

In view of the difficulty of exactly evaluating acetylesterase at light microscopy, we processed specimens for ultrastructural evaluation after enzymatic staining with AChE (Figure 5a). The results showed that the reaction product was localized both in the basal portion of the gustatory epithelium and in the connective layer below taste buds.

Ultrastructural control of peptidergic fibers distribution

Ultrastructural immunocytochemistry revealed CGRP-IR axons localized both in the basal portion of the gustatory epithelium and in the connective layer below taste buds. In addition, CGRP-IR as well as SP fibers were also found in perigemmal position (Figure 5b).

Discussion

A large body of literature has described the presence and the immunocytochemical characteristics of nerve fibers in the gustative epithelium (Nagy *et al.*, 1982; Hirata and Kanaseki, 1989; Farbman and Mbiene, 1991; Montavon and Lindstrand, 1991; Mbiene and Farbman, 1993; Nelson and Finger, 1993; Huang and Lu, 1996; Kanazawa and Yoshie, 1996; Kusakabe *et al.*, 1998). Recently, however, new data concerning the presence of an intrinsic nervous system in the gustative epithelium (Hu *et al.*, 1996; Sbarbati *et al.*, 2002) have given rise to new questions not only about the distribution, but also about the neurochemical composition of these fibers. To our knowledge, this is the first report to demonstrate that the plexus consists of two layers, superficial and deep; each layer is well-characterized neurochemically. The immunocolocalization results that we obtained using confocal microscopy demonstrate that the two systems of fibers are spatially separate and that the neurochemical composition of the two nerve layers is different. The superficial layer is composed of peptidergic and nitrergic nerve fibers; the latter are located below the basal lamina of the chemoreceptorial epithelium and within the boundaries of the taste buds. Some of the immunoreactive nerve processes appear to be perigemmal or intragemmal. The deeper layer is composed of nitrergic and vipergic nerve fibers, which were not in direct contact with the buds and ran below the superficial layer.

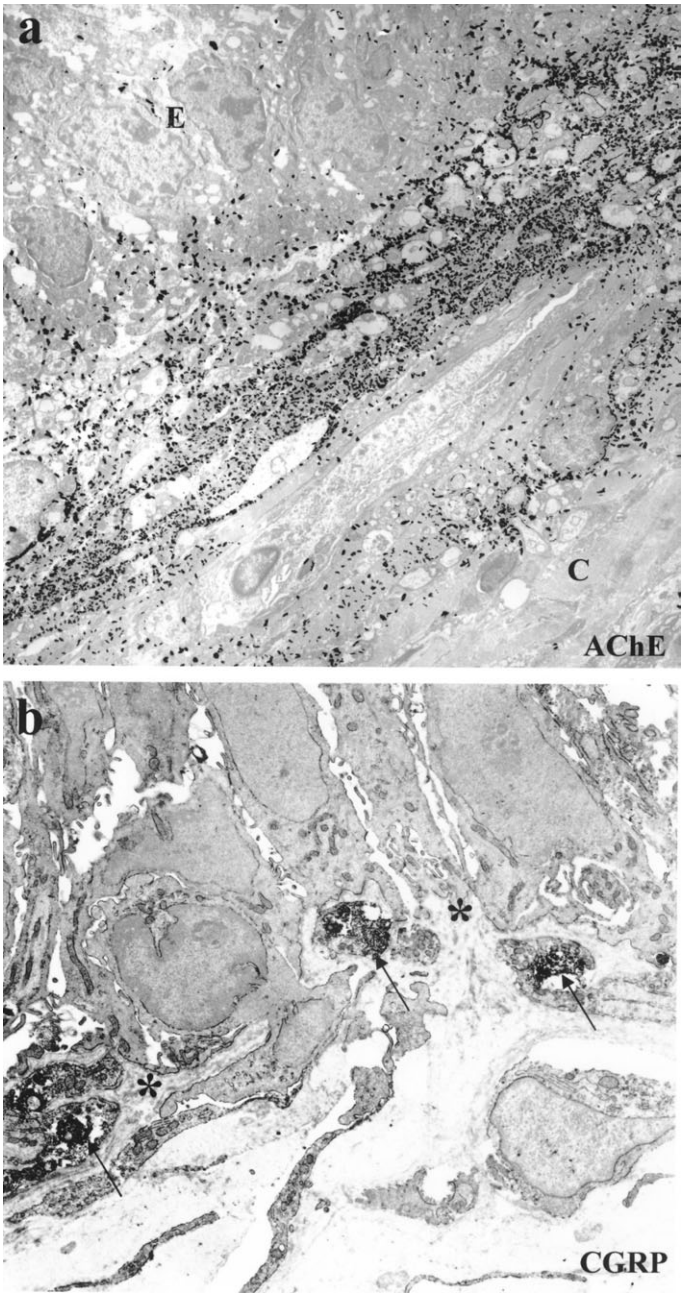

Figure 5 Ultrastructural cytochemistry for AChE and immunocytochemical distribution of peptidergic fibers. **(a)** Ultrastructural enzymatic staining for AChE shows that the reaction product is extracellular and localized both in the basal portion of the gustatory epithelium and in the connective layer below the taste buds. E, epithelium; C, connective. **(b)** Ultrastructural immunocytochemistry reveals CGRP-IR axons localized in the basal portion of the gustatory epithelium and in the connective layer below the taste buds (arrows). In addition, CGRP-IR as well as SP fibers were also found in perigemmal position; basal membrane is marked by asterisk. Scale bars = 4 μm (a); 2.5 μm (b).

The superficial subgemmal layer

The superficial layer is in direct contact with the chemoreceptorial epithelium and the fibers of this layer penetrate into the epithelium, contacting taste cells. Our data show that this (external) layer can be marked by CGRP, SP, CCK and AChE. These bundle of fibers are both composed of intragemmal afferent fibers, directly involved in taste and of perigemmal fibers. The presence of CCK fibers in these

layers should be emphasized. Fibers containing CCK have already been shown to be present in the tongue (Herness *et al.*, 2002), but their role is not clear. Their presence in the superficial layer suggests direct involvement in tasting processes.

Ultrastructural data clarified that the superficial layer and the intragemmal nerve fibers showed similar neurochemical characteristics, demostrating that the two zones are anatomically and neurochemically continuous.

The deeper subgemmal layer

The inner layer (deeper) fibers are not in direct contact with the chemoreceptorial epithelium and they are characterized by the presence of fibers positive for VIP, NOS-1 and NF-200. This layer is also associated with intrinsic neurons which are in large part nitrergic elements.

These findings suggest that the possible modulatory effects on the taste cells operated by the nitrergic system are not due to direct contact, because of the absence of intra-epithelial nitrergic fibers. It is, however, possible that axo-axonal contact exists at the interface between the two subgemmal layers. In addition, our data demonstrate that NF-200 antibody represents an effective way to evaluate the intrinsic nervous system. Extrinsic fibers (CGRP positive) were not stained by NF-200 antibody. This finding seems to be in agreement with other studies demonstrating the presence in rats of axons which contain only NF-L and NF-M (Balin and Lee, 1991). Yet it is interesting to observe that NF-200 immunoreactive fibers exactly colocalize with NADPH-diaphorase fibers; in the light of this, it is possible to assert that NF-200-IR fibers are intrinsic fibers.

Our results also demonstrate the complexity of the peptidergic system. Fibers positive for CCK, CGRP and SP are distributed in the superficial layer, whereas VIP-IR fibers are spatially distributed in the deep layer and associated with the nitrergic fibers, so that the relationship with the chemoreceptorial epithelium appears less direct. A role in processes ancillary to taste, i.e. control of vascular or secretory mechanisms, seems probable for this peptidergic component.

General conclusion

The present work demonstrates that the basal plexus is a bilayered structure. Further studies are necessary to clarify the reciprocal relationship between the different components and to complete the chemical coding of the intrinsic neurons associated with the deeper layer.

Our previous studies (Sbarbati *et al.*, 2000, 2002) suggest that the intrinsic nervous system (INT) of the circumvallata papilla may be the rostral member of the enteric nervous system (ENS), representing a kind of chemical eye, in that it is in a position to supply a chemical analysis of foods as they enter the alimentary canal. If this hypothesis is correct, the intrinsic nervous system of the circumvallata papilla would have an organization and complexity similar to that of other structures in the enteric nervous system—that is, a subdivision of the neurons into subgroups and a neurochemical organization of the fibers. Previously, we demonstrated that different subpopulations of neurons are located in this organ (Sbarbati *et al.*, 2002). The present study shows a clear distinction between fibers associated with the taste epithelium and the possibility of describing neurochemically distinct areas. Therefore, the complex chemical coding of taste seems to be similar to the neurochemistry of the ENS, strengthening the idea that the INT of the papilla is the cephalic member of the ENT. Indeed, future studies are necessary to understand the morpho-functional relations between these two structures.

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