Anat Embryol (2001) 204:161–169

ORIGINAL ARTICLE

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The distribution of PGP9.5, BDNF and NGF in the vallate papilla of adult and developing mice

Accepted: 26 April 2001

Abstract The development and innervation of vallate papillae and taste buds in mice were studied using antibodies against the neuronal marker, protein gene product 9.5 (PGP 9.5), and against nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). PGP 9.5 immunohistochemical studies revealed that the earliest sign of median vallate papilla formation was an epithelial bulge at embryonic day 13 (E13), and at E14, a dense nerve plexus was found within the connective tissue core of the papilla. Thin nerve fibers penetrated the apical and medial trench wall epithelium of the papilla at E16 and a few of these began to invade the lateral trench wall epithelium at E17. At postnatal day 1 (P1), the newly formed taste buds were recognizable and a small number of PGP 9.5-immunoreactive (IR) cells appeared on the medial trench wall epithelium. The number of PGP 9.5-IR taste bud cells then increased gradually and reached the adult level at postnatal week 2. PGP 9.5 immunoreactivity increased systematically with age. NGF and BDNF immunoreactivity was first seen at the boundary between the columnar cells in the apical epithelium of the developing vallate papilla at E13, then in the medial and lateral trench walls at E15 (BDNF) or E18 (NGF). At P1, BDNF immunoreactivity was exclusively present in the newly formed taste buds of the medial trench wall. The number of BDNF-IR taste bud cells then increased gradually, reaching the adult level at P7. Similar degrees of NGF and BDNF immunoreactivity were seen in the developing vallate papilla. In the present study, we found that the vallate papilla was formed prior to its innervation, and we propose that initiation of papilla formation does not require any direct influence from the specific gustatory nerve. We also suggest that neurotrophins in the early developing vallate papillae might act as local tropic factors for the embryonic growth of nerve fibers to induce differentiation of the taste buds.

Keywords Taste bud · Neurotrophins · Development · Innervation · Immunohistochemistry

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Introduction

Taste buds, the chemoreceptor end organs for taste, are clustered in the lingual papillae, especially the vallate papilla, and are distributed diffusely over the extralingual areas. They consist of spindle-shaped taste receptor cells that are considered to be neuroepithelial in nature and to arise from the local epithelium during development (Farbman 1965; Zalewski 1974; Barlow and Northcutt 1995; Stone et al. 1995). The arrival of the nerve supply in the embryonic gustatory epithelium has been shown to precede the appearance of the taste buds (Torrey 1940; Farbman 1965) and damage to the glossopharyngeal nerve at an early stage of development results in the loss of taste buds in the vallate papillae (Hosley et al. 1987a,b). This evidence supports the hypothesis that the gustatory nerve induces differentiation of taste buds and is responsible for the structural and functional maintenance of taste buds in vertebrates.

The protein-gene product 9.5 (PGP 9.5) is found predominantly in the cytoplasm of neurons and neuroendocrine cells and is a pan-neuronal marker for nervous tissue (Thompson et al. 1983). Antibodies against PGP 9.5 have been used to demonstrate nerve fibers in adult and developing peripheral tissues, including the rat tooth (Fristad et al. 1994; Luukko 1997), mouse vallate papillae (Wakisaka et al. 1996), and hamster olfactory bulb (Nakajima et al. 1998).

Nosrat and Oslon (1995) proposed that the capacity for differentiation into taste buds is probably related to the ability of the gustatory epithelium to express a specific neurotrophic factor and the same group demonstrated by in situ hybridization that brain-derived neurotrophic factor (BDNF) mRNA is expressed in a specific

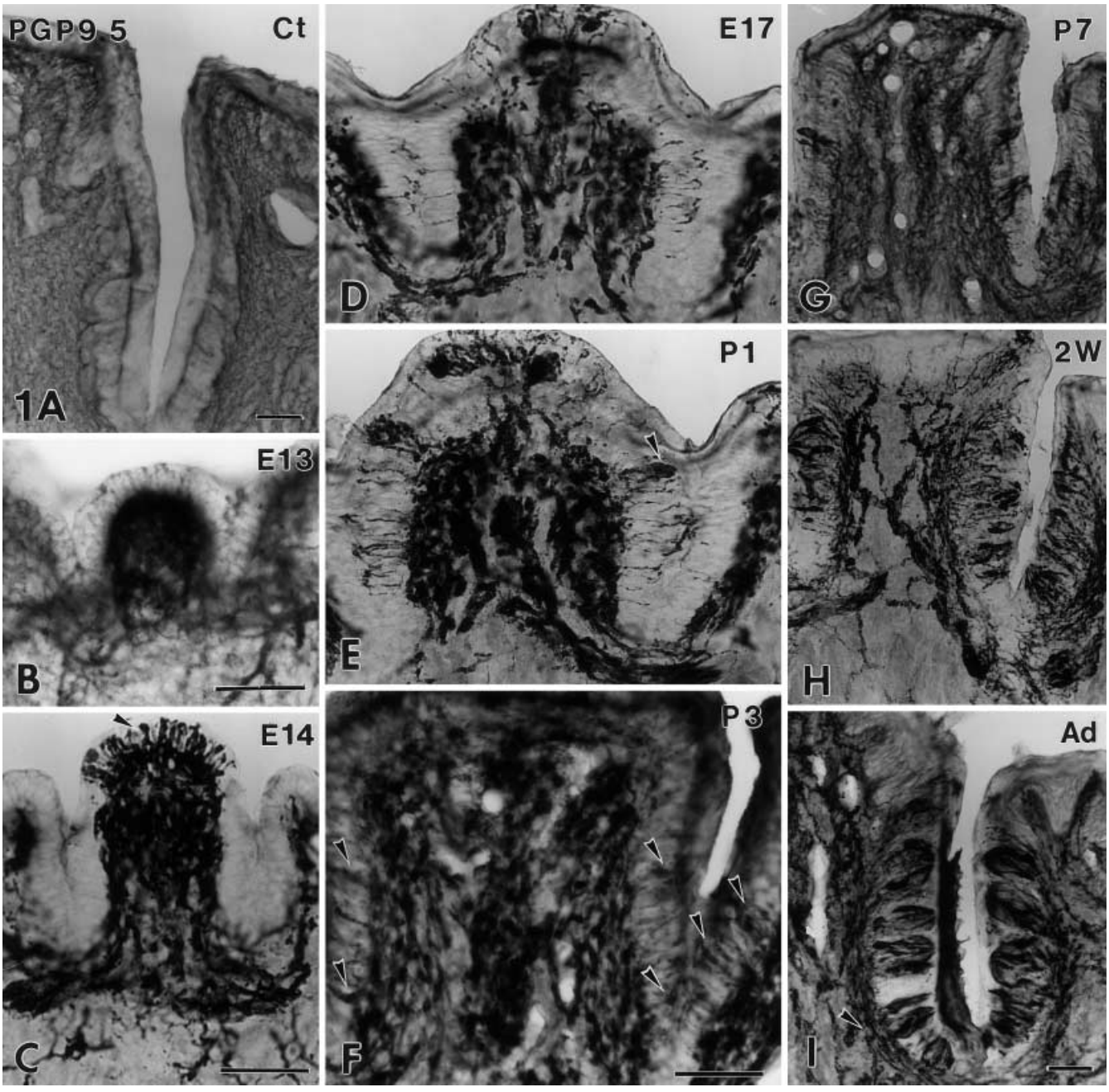

Fig. 1 PGP 9.5 immunoreactivity in mouse vallate papillae at adult with negative control (**A**) and at E13 (**B**), E14 (**C**), E17 (**D**), P1 (**E**), P3 (**F**), P7 (**G**), 2 weeks (**H**), and adult (**I**). At E13 (**B**), thick nerve bundles below the newly forming papilla and in the lamina propria beneath the papillary core display PGP 9.5 immunoreactivity, which is never found in the trench wall epithelium at this stage. Thin PGP 9.5-IR nerve fibers invade the apical epithelium at E14 (**C**), and a few round-shaped cells also show PGP 9.5 immunoreactivity (*arrowhead*). Both medial and lateral trench walls are penetrated by the PGP 9.5-IR nerve fibers at E17 (**D**). The oval-shaped PGP 9.5-IR cells (*arrowhead*) are observed in the medial trench wall at P1 (**E**), and detected in the lateral trench wall at P3 (**F**). The immunoreactivity of PGP 9.5-IR nerve fibers and cells increases in the taste buds in the trench wall epithelium from P3 (**F**) to P7 (**G**). At 2 weeks postnatal (**H**), PGP 9.5 immunoreactivity in the vallate papilla is similar to that in the adult (**I**). In the adult vallate papilla, the PGP-IR nerve fibers form subgemmal nerve plexus below the taste buds (*arrowhead*). These nerve fibers penetrate the taste buds as intragemmal fibers and between the taste buds as extragemmal nerve fibers. PGP 9.5 immunoreactivity is present in spindle-shaped taste bud cells in the taste buds. Bars **A**, **G–I** 100 μm; **B–F** 100 μm

pattern in the taste bud cells of the developing and adult rat fungiform and vallate papillae (Nosrat et al. 1996). Studies by various investigators on BDNF null mutant mice have indicated that, during taste bud development, either the absence or ectopic over-expression of BDNF causes sparse innervation and reduces the gustatory epithelium area and the number of taste buds in the vallate papilla (Nosrat et al. 1997; Zhang et al. 1997; Oakley et al. 1998; Mistretta et al. 1999; Ringstedt et al. 1999). The development of gustatory papillae requires appropri-

ate neurotrophic factors for appropriate target invasion and innervation.

In order to investigate the development of the taste bud with special emphasis on its neural function, we studied the innervation patterns of vallate taste buds in developing and adult mice using immunohistochemical methods and antibodies against PGP 9.5. In addition, although many studies have investigated the function of neurotrophins using gene-knockout mice, to the best of our knowledge, no direct studies on the distribution patterns of the neurotrophins, BDNF and NGF have been reported. We therefore determined the immunohistochemical localization of these two neurotrophins in the vallate papilla in developing and adult mice to study the relationship between neurotrophins and neuronal events during taste bud development.

Materials and Methods

Animals. Adult ICR mice were housed in a temperature-controlled room (22±1°C)under artificial illumination (lights on from 05:00 h to 17:00 h) and at 55% relative humidity, with free access to food and water. Prenatal and postnatal mice were obtained from time-mated pregnant mice. The day that the presence of the vaginal plug was confirmed was designated embryonic day 0 (E0) and the day of birth was designated postnatal day 0 (P0). Embryos at various development stages (E12–18, daily intervals) and neonatal animals at various postnatal stages (P0, 1, 2, 3, 4, 5, 6, 7, 10, 12, 2w, 3w, 4w, 6w, 8w, and 10w) were used in the present study.

Tissue preparation. Pregnant and postnatal mice were anesthetized swith sodium pentobarbital (30 mg/kg body wt., i.p.). The fetuses were removed from the uteruses of pregnant females under deep anesthesia. Prenatal and early postnatal (up to P7) mice were killed by decapitation, while animals at later stages were killed by perfusion through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently the tongues were excised, and small blocks containing the vallate papillae were cut out and fixed at 4°C for 24 h. After cryoprotection with successively increasing concentrations of sucrose (10%, 20%, and 30% in 0.1 M phosphate buffer), tissue sections (20 µm) were prepared on a cryostat. All procedures were approved by the Animal Care and Use Committee of our university.

Immunohistochemistry. Immunostaining was performed on free-floating cryostat sections with indirect immunofluorescence or immunoperoxidase visualization. The sections were first pre-incubated for 2 h at room temperature in 0.1 M phosphate-buffered saline (PBS) containing 10% normal goat serum (NGS) and 0.3% H_2O_2 to block endogenous peroxidase activity and nonspecific binding of antibodies before being incubated for 20 h at 4°C with primary rabbit antibodies against PGP 9.5 (1:1,000; Biogenesis, UK), BDNF, or NGF (both 1:200; Santa Cruz, USA), diluted in PBS. For immunofluorescence staining, the sections were then treated for 1 h at room temperature with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200; Cappel, Durham, N.C., U.S.A.), and mounted with crystal/mount (Biomeda, U.S.A.). For immunoperoxidase staining, sections were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (1:100, Vector, U.S.A.), then with the reagents from an ABC kit (Avidin-Biotin Complex, Vector Laboratories, U.S.A.), following the manufacturer's recommendations, and the reaction products visualized by incubation for 2–3 min at room temperature with 0.5 mg/ml of 3,3-diaminobenzidine, 0.003% H_2O_2 in 0.5 M TRIS buffer, pH 7.6 before the sections were mounted on gelatin-coated slides using Permount (Fisher, U.S.A.). All immunostained sections were viewed and photographed using a Zeiss Axiophot microscope equipped with an epifluorescence attachment.

Dot immunoassay. To test the specificity of the antiserum, dot immunoassay was performed for anti-BDNF and anti-NGF antibodies (1:200) with their respective antigens (both 200 µg/ml; Santa Cruz, CA, U.S.A.), on the separate stripes of nitrocellulose membrane for 2 h at 37°C. A cross-reactivity dot immunoassay was also carried out for anti-BDNF and anti-NGF with antigens NGF and BDNF respectively. After binding of antigen and antibody, the TBS buffer washed nitrocellulose membrane was then incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and rendered to blue colour with a combination of nitro blue tetrazolium (NBT, Sigma, Mo., USA) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Sigma) in alkaline phosphatase assay buffer. For the controls, sections were incubated in medium containing pre-absorbed primary antiserum or medium without primary antiserum, immunostaining of samples being regarded as specific when no immunostaining was seen in these controls.

Results

The neuronal marker PGP 9.5

The control sections from adult mice (Fig. 1A), in which primary antibody was omitted, showed only nonspecific weak background staining. At E12–13, slight bulging of the surface epithelial cells on the midline of the posterior tongue was seen, suggestive of vallate papilla formation. Abundant PGP 9.5-IR nerve fibers appeared in the basal part of the papilla (Fig. 1B), ascended in the lamina propria to the basal part of the apical epithelium, and formed dense nerve plexuses in the lamina propria of the papilla.

At E14–E16, the trench of the papilla became deeper and the vallate papilla formed. At E14 (Fig. 1C), many thin PGP 9.5-IR nerve fibers penetrated into the apical epithelium from the nerve plexuses in the lamina propria and ramified perpendicularly to the epithelial surface. A few round PGP 9.5-IR cells were seen in the apical epithelium. At E17, PGP 9.5-IR nerve fibers penetrated the epithelium of the trench wall (Fig. 1D). More nerve fibers were seen in the medial trench wall than in the lateral trench wall.

In newborn animals (Fig. 1E), oval PGP 9.5-IR cells were seen in the medial trench wall epithelium of the vallate papilla. Two days later (Fig. 1F), PGP 9.5-IR cells were also seen in the lateral trench wall. The num-

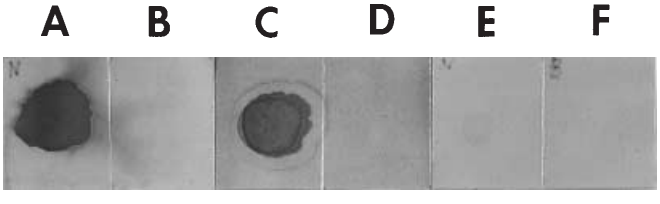

Fig. 2A–F Dot-binding immunoassay. NGF (**A**) and BDNF (**C**) were directly dotted onto membrane strip as positive controls, or TBS-Tween (**B**, **D**) as negative controls. The membrane stripes are then treaated with anti-NGF (**A**, **B**) or anti-BDNF (**C**, **D**) antibody. Binding between anti-NGF (**A**) and NGF or anti-BDNF (**C**) and BDNF was tested. Cross reactivity between NGF (**E**) and BDNF (**F**) with anti-BDNF antibody and anti-NGF antibody, respectively, was also examined

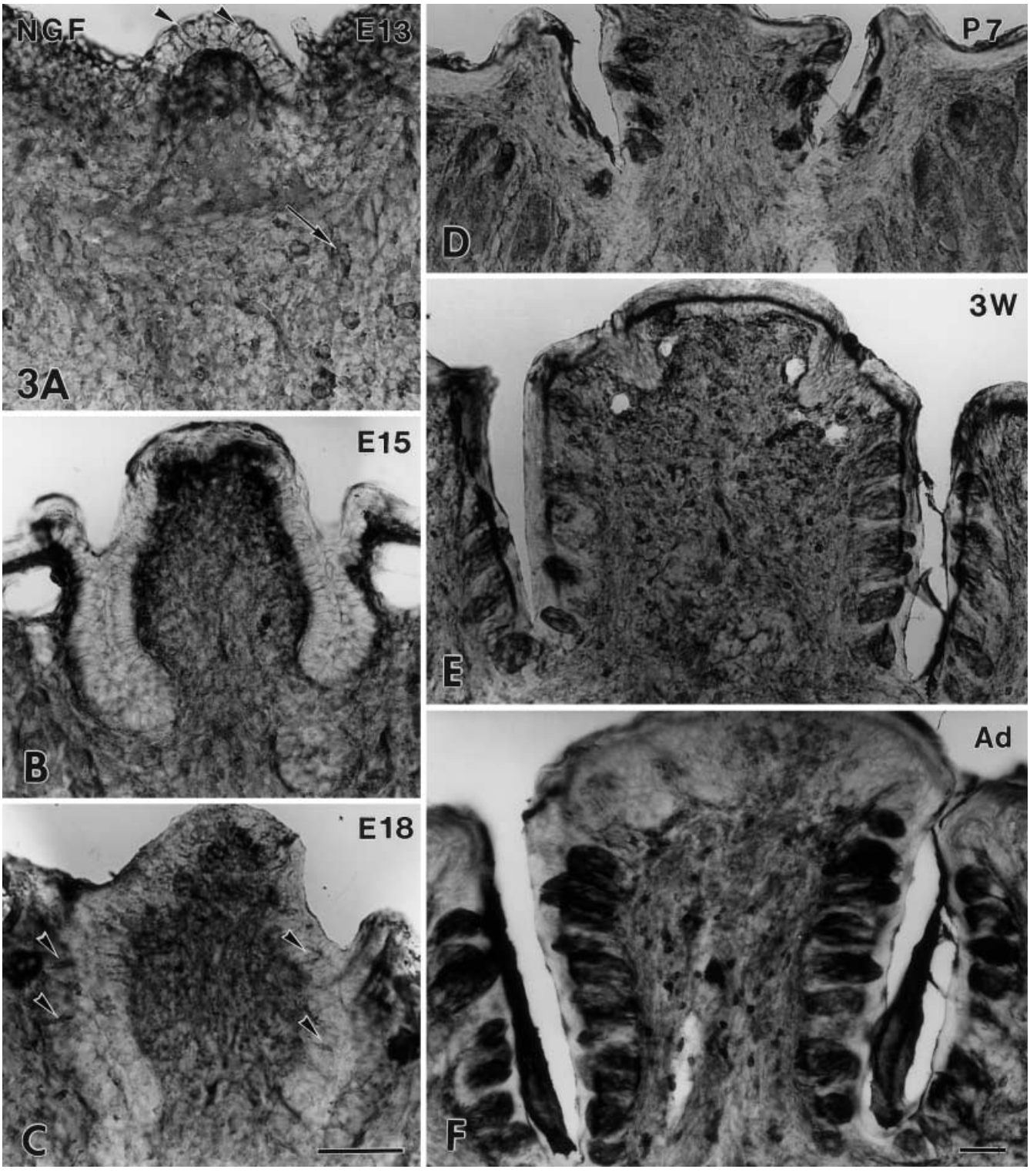

Fig. 3A–F Immunoperoxidase staining for NGF in mouse vallate papillae at E13, E15, E18, P7, 3 weeks, and adult. NGF immunoreactivity is first seen at the edge of the apical epithelial cells (*arrowhead*) and fusiform cells in the deeper layer of the tongue (*arrow*) at E13 (**A**), and is more distinct at E15 (**B**). Both the medial and lateral trench walls show NGF-IR cytoplasm (*arrowhead*) in the E18 vallate papilla (**C**). At P7 (**D**), the immunoreactivity is similar to that seen in the adult (**F**) and is present in almost all of the taste buds. The number of stained taste buds increases progressively, then remains constant from week 3 (**E**) to the adult (**F**). Bars **A–C** 100 μm; **D–F** 100 μm

ber of PGP 9.5-IR nerve fibers in the trench wall epithelium of vallate papilla increased gradually from P0 to P5. At P0-P1, taste buds were recognizable within the epithelium of both the lateral and medial trench walls, extending from the basal lamina to the free surface of the trench wall epithelium.

At P6 (Fig. 1G), the morphology and PGP 9.5 immunoreactivity of the vallate papilla resembled those in the adult. After P6, the number of PGP 9.5-IR cells in the

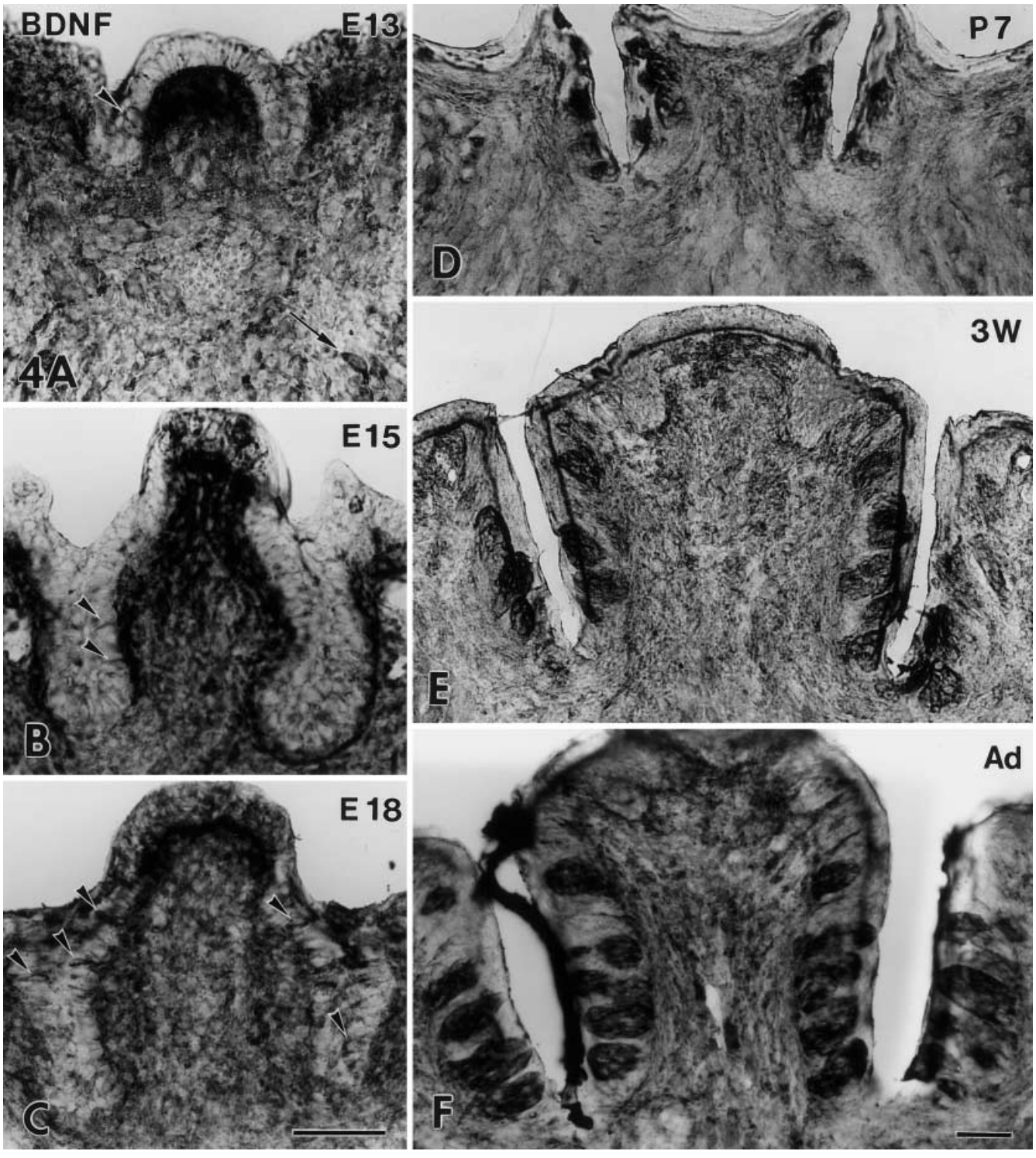

Fig. 4A–F Immunoperoxidase staining for BDNF in mouse vallate papillae at E13, E15, E18, P7, 3 weeks, and adult. BDNF immunoreactivity is seen at the edge of the apical epithelial cells (*arrowhead*) and fusiform cells in the deeper layer of the tongue (*arrow*) at E13 (**A**). The immunoreactivity is more distinct in the apical epithelial cells and is less intensively expressed in the cytoplasm (*arrowhead*) in both the medial and lateral trench walls at E15 (**B**). At E18 (**C**), the immunoreactivity of vallate papilla becomes stronger and more extensive (*arrowhead*). All taste buds at P7 (**D**) are immunoreactive, the intensity of staining then remaining constant through week 3 (**E**) to the adult (**F**). The distribution pattern is also similar from P7 to the adult. Bars **A–C** 100 µm; **D–F** 100 µm

taste buds and the density of PGP 9.5-IR nerve fibers in the connective tissue core of the vallate papillae apparently increased with age, although systematic counts were not performed. At postnatal week 2 (Fig. 1H), the numbers of PGP 9.5-IR taste bud cells and nerve fibers in the vallate papilla were similar to those in the adult (Fig. 1I). In adult animals, dense PGP 9.5-IR fiber bundles in the connective tissue beneath the vallate papillae were directed towards the trench wall where the taste buds were located, and PGP 9.5-IR fibers were seen be-

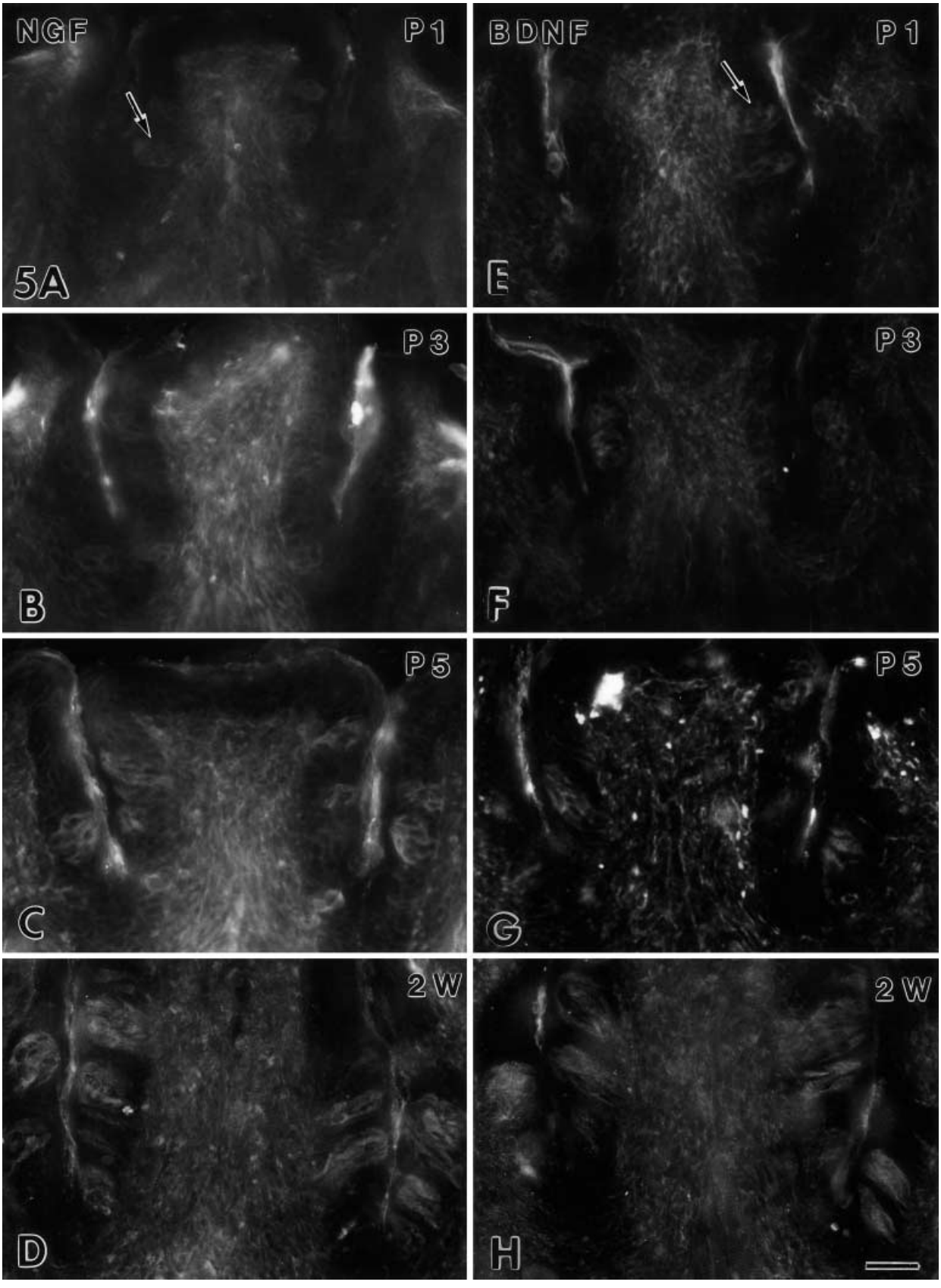

Fig. 5A–H Immunofluorescence staining for NGF and BDNF in mouse vallate papillae at P1, P3, P5, and 2 weeks. **A–D**: NGF; **E–H**: BDNF. Immunoreactivity is present in the cells of the primordial taste buds (*arrow*) and is more intense in the medial trench walls at P1 (**A**, **E**) then gradually increases in the lateral trench walls from P3 (**B**, **F**) to P5 (**C**, **G**). The intensity of the immunoreactivity and the number of immunoreactive taste buds in the vallate papillae at 2 weeks are similar to those in the adult (**D**, **H**). Bar **A–H** 100 μm

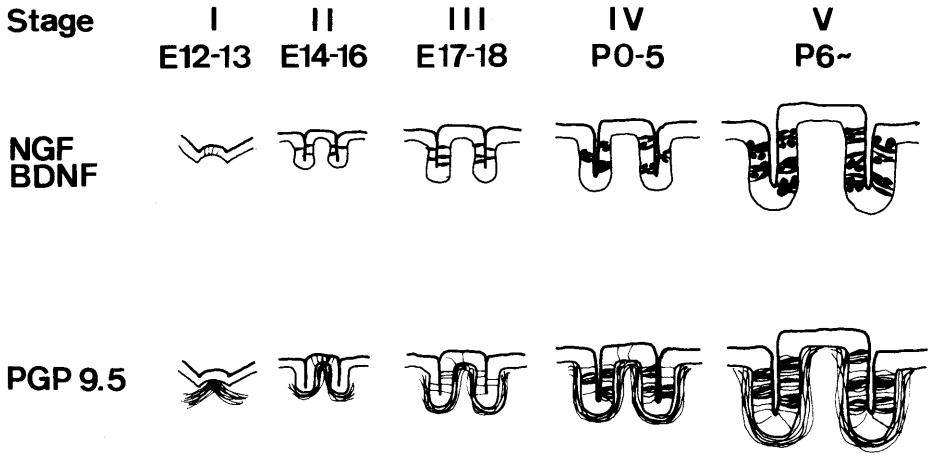

Fig. 6 Schematic diagrams showing the development of BDNF- and NGF-IR (*upper row*) and PGP 9.5-IR (*lower row*) neuronal structures in the mouse vallate papilla. The solid lines indicate immunoreactive nerve fibers and the dark spots immunoreactive taste cells

low the trench wall (subgemmal nerve plexus), penetrating the taste bud (intragemmal nerve fibers) or the epithelium between the taste buds (extragemmal nerve fibers).

The neurotrophic factors NGF and BDNF

The result of dot immunoassay revealed that the anti-NGF (Fig. 2A) and anti-BDNF (Fig. 2C) antibodies bind to the respective antigen specifically. No binding was observed in the negative controls (Fig. 2B, D) and in the cross reactivity test (Fig. 2E, F).

Basically, a similar pattern of NGF and BDNF immunoreactivity was seen in developing, young, and adult mice. However, before P5, the intensity of immunofluorescence or DAB staining for NGF was stronger than that for BDNF, whereas, after P5, equivalent immunostaining intensity was seen for the two neurotrophins.

At E12–E13, NGF-IR (Fig. 3A) and BDNF-IR (Fig. 4A) fusiform cells could be seen in the deeper layer of the embryonic tongue, as well as at the edge of the apical epithelial cells of the vallate papilla. At E15, faint BDNF (Fig. 4B), but not NGF (Fig. 3B), immunoreactivity could be seen in the epithelium of the trench wall, the reaction products being located in the cytoplasm of the epithelial cell. At E17–E18, the number of fusiform NGF-IR (Fig. 3C) and BDNF-IR (Fig. 4C) cells increased gradually in the epithelium of the trench wall.

From P1 (Fig. 5A, E) to P3 (Fig. 5B, F), NGF-IR and BDNF-IR cells in the taste bud were more intensely stained in the medial trench wall than in the lateral trench wall. Occasionally, NGF-IR and BDNF-IR cells were observed in the connective tissue core of the vallate papilla. At P7, the staining intensities of the NGF-IR (Fig. 3D) and BDNF-IR (Fig. 4D) taste cells were similar to those seen in the adult. In addition, the number of taste buds increased with age (Figs 3E, F, 4E, F, 5D, 5H).

In the adult, most of cells in the taste buds of the vallate papilla appeared intensely immunostained for NGF and BDNF. Nerve fibers in the trench walls and connective tissue core of vallate papillae were not obviously immunostained with anti-NGF and anti-BDNF antibodies. The distribution of NGF and BDNF immunoreactivity in the mouse vallate papilla was almost identical.

Figure 6 summarizes the results of the present study and shows the detailed distribution of PGP 9.5, NGF, and BDNF immunoreactivity in the vallate papilla in developing and adult mice.

Discussion

PGP 9.5

PGP 9.5, a neuronal cytoplasmic protein and pan-neuronal marker, has been widely used to visualize different populations and subtypes of nerves (Thompson et al. 1983). It is a good marker for fine peripheral nerve fibers and has been extensively used for the study of lingual and gustatory papillae innervation (AhPin et al. 1989; Wakisaka et al. 1996, 1998; Mbiene and Mistretta 1997; Ringstedt et al. 1999). Wakisaka et al. (1996) reported the presence of PGP 9.5-IR nerve fibers and neurons in the vallate papilla in the developing mouse. In the present study, PGP 9.5 immunoreactivity was also used to provide information on the development and innervation of the vallate taste bud in adult and developing mice. Our observations on the occurrence and distribution of PGP 9.5 immunoreactivity in the connective tissue core and in the epithelium of vallate papilla were essentially similar to those of Wakisaka et al. (1996). However, minor differences were seen. The invasion of the lateral trench wall by PGP 9.5-IR nerve fibers was seen as early as E17 and PGP 9.5-IR taste cells were seen in the medial trench wall at P1, in both cases earlier than the corresponding dates of P0-P3 and P5-P10 reported by Wakisaka et al. (1996). In addition, our results correlated well with those of AhPin et al. (1989), who showed that the invasion of the apical epithelium of the vallate papilla by nerve fibers occurs at E14 in Balb/c mice (demonstrated by silver impregnation) and that the appearance of oval or round PGP 9.5-IR cells in the apical epithelium coincides with penetration of PGP 9.5-IR nerve

fibers into the apical epithelium. Since no apparent taste buds were recognized in the apical epithelium in mature vallate papilla, we propose that these PGP 9.5-IR cells may represent Merkel-type cells as suggested by Wakisaka et al. (1996).

BDNF and NGF

The trophic dependence of taste buds on the influence of gustatory or nongustatory nerve branches has been known for many years (Torrey 1940; Wright 1955; Hosley et al. 1987a, b), but the reason why nerve fibers are involved in the differentiation of taste bud is still unknown. The dependence of the early primordial taste bud on nerves has recently been questioned, as, in ectopic epithelia, taste buds develop without their proper nerve supply (Barlow and Northcutt 1995; Barlow et al. 1996).

BDNF and NGF mRNAs have been detected in developing taste bud-bearing tongue papillae (Nosrat and Olson 1995; Nosrat et al. 1996). These authors demonstrated that BDNF mRNA is expressed in the epithelium of the superior and posterior surface of the papillae at E15, E16, and E17, starts to decrease in the superior epithelium as early as E17, and, at E19 and E21, is found exclusively in the epithelium of the inner and outer walls of the trench, surrounding the papilla at the posterior and lateral surface where the taste buds are located later in life. However, in their studies, the amount of NGF mRNA was below the detection level and BDNF mRNA was first detected at the superior surface of the rat vallate papilla at E15, the day of initiation of papilla formation in the rat. In the present study in mice, BDNF and NGF immunoreactivity was seen in the apical surface of the mouse vallate papilla at E13–14, 1–2 days after the initiation of vallate papilla formation in this species (E11–12), and only BDNF was seen in the trench walls at E15–16, much earlier than the first appearance of BDNF mRNA in the anterior and posterior epithelium of the rat vallate papilla reported by Nosrat and Olson (1995). The result that only BDNF was seen in the trench wall coincides with the results of Nosrat and Olson (1995) and Nosrat et al. (1996). The discrepancy in these results may be due to species difference or the techniques used (in situ hybridization versus immunofluorescence). Despite the species difference we suggest that BDNF mRNA is expressed in the initiation stage of the vallate papilla formation and that BDNF immunoreactivity is observed 1–2 days after the formation of vallate papilla.

Since transgenic mice overexpressing BDNF have lingual gustatory deficits similar to those seen in BDNF null mutant mice (Nosrat et al. 1997; Zhang et al. 1997; Oakley et al. 1998; Mistretta et al. 1999), Ringstedt et al. (1999) suggested that BDNF acts as a target invasion factor for early arriving gustatory fibers in the tongue and coordinates innervation of the correct targets. Cooper and Oakley (1998) found that not all taste neurons died in BDNF null mutation mice (–/–), and suggested that the surviving BDNF-deprived taste neurons were rescued by a redundant neurotrophic factor at the level of the local gustatory epithelium.

Kessler and Black (1981) demonstrated that, in the dorsal root ganglion and spinal cord of the intrauterine forelimb amputation rat, NGF treatment increases substance P (SP) levels, and suggested that NGF could regulate putative transmitter development within the CNS, as well as in peripheral structures. NGF overexpressing transgenic mice also show an age-dependent induced novel hyperinnervation of vallate papillae by tyrosine hydroxylase-containing nerve fibers (Takami et al. 1996). Moreover, the result of Crowley et al. (1994) that mice with a deletion in the coding sequence of the NGF gene have cell loss in both sensory and sympathetic ganglia confirms the critical dependence of sensory and sympathetic neurons on NGF, which cannot be compensated for by other neurotrophins. In addition, NGF is taken up from the periphery by nerve fibers and transported to the soma (Stoeckel et al. 1975), where it may up-regulate both SP and CGRP expression and stimulate neurite outgrowth (Yasuda et al. 1990). Taking all these results together, it is reasonable to speculate that the NGF seen in prenatal and postnatal ICR mice is probably redundant and preparing to rescue the BDNF deprived taste neuron.

Barlow et al. (1996) demonstrated that taste cells differentiate fully in the complete absence of innervation; when the presumptive oropharyngeal region was taken from a donor axolotl embryo, prior to innervation and the development of taste buds, and grafted ectopically on to the truck of a host embryo, the graft developed well-differentiated taste buds. In our study, BDNF and NGF immunoreactivity was detected in the gustatory epithelium before the appearance of PGP-IR nerve fibers, indicating that nerve invasion may result from the action of neurotrophins within the epithelium. During the turnover of taste cells in mature taste buds in adult rats, the degenerating taste cells exhaust the neurotrophins and the nerves withdraw. In contrast, during the renewal of taste cells, both BDNF and NGF may attract nerves to the differentiating taste cells.

In summary, we demonstrated the expression of BDNF and NGF during the development of the mouse vallate papilla and their taste buds, and we speculate that neurotrophins may promote the differentiation of nerve fibers and the subsequent maintenance of the taste buds. Further studies to correlate these findings with the ultrastructural analysis are in progress.

Acknowledgements This work was supported in part by grant no. NSC-89–2320-B002–279 from the National Science Council, Taiwan. The technical assistance of Mr. B. N. Huang and Ms. S. M. Lai is greatly appreciated. We should also like to express our sincere appreciation to Dr. Thomas Barkas for revising the English of the manuscript.

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