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Epithelial Overexpression of BDNF or NT4 Disrupts Targeting of Taste Neurons That Innervate the Anterior Tongue

Robin F. Krimm,*[,1] Kristy K. Miller,* Patrick H. Kitzman,* Brian M. Davis,† and Kathyrn M. Albers*[,†]

**Department of Pathology and Laboratory Medicine and †Department of Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, Kentucky 40536-0298*

Brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4) are essential for the survival of geniculate ganglion neurons, which provide the sensory afferents for taste buds of the anterior tongue and palate. To determine how these target-derived growth factors regulate gustatory development, the taste system was examined in transgenic mice that overexpress BDNF (BDNF-OE) or NT4 (NT4-OE) in basal epithelial cells of the tongue. Overexpression of BDNF or NT4 caused a 93 and 140% increase, respectively, in the number of geniculate ganglion neurons. Surprisingly, both transgenic lines had severe reduction in fungiform papillae and taste bud number, primarily in the dorsal midregion and ventral tip of the tongue. No alterations were observed in taste buds of circumvallate or incisal papillae. Fungiform papillae were initially present on tongues of newborn BDNF-OE animals, but many were small, poorly innervated, and lost postnatally. To explain the loss of nerve innervation to fungiform papillae, the facial nerve of developing animals was labeled with the lipophilic tracer DiI. In contrast to control mice, in which taste neurons innervated only fungiform papillae, taste neurons in BDNF-OE and NT4-OE mice innervated few fungiform papillae. Instead, some fibers approached but did not penetrate the epithelium and aberrant innervation to filiform papillae was observed. In addition, some papillae that formed in transgenic mice had two taste buds (instead of one) and were frequently arranged in clusters of two or three papillae. These results indicate that target-derived BDNF and NT4 are not only survival factors for geniculate ganglion neurons, but also have important roles in regulating the development and spatial patterning of fungiform papilla and targeting of taste neurons to these sensory structures.

Key Words: **neurotrophin; BDNF; NT4; taste bud; geniculate ganglion; fungiform papillae; target selection.**

INTRODUCTION

The peripheral anatomy of the taste system makes it useful for examining neuronal–target interactions during development. Taste buds on the mammalian tongue are located within complex structures called papillae that are distributed across the tongue in a defined spatial pattern (Mistretta, 1991). Within papillae, the location and number of taste buds is very predictable. For example, in rodents, fungiform papillae are spaced in a fairly even array across the front two-thirds of the tongue (Miller and Preslar, 1975), and at the center apex of each papilla is one taste bud. Thus, papillae and taste buds provide discrete, predictable targets for their innervating sensory neurons.

The tongue epithelium is highly selective for either gustatory or somatosensory innervation. Receptor cells of taste buds are innervated by gustatory neurons of the geniculate ganglion via the chorda tympani nerve, while the remaining epithelium, which includes nontaste filiform papillae, is innervated by somatosensory neurons of the trigeminal ganglion. During initial tongue innervation, nerves follow precise, spatially restricted pathways, suggesting neural guidance is regulated by molecular cues from the environment (Mbiene and Mistretta, 1997; Rochlin and Farbman, 1998; Rochlin *et al.*, 2000). Gustatory nerve fibers appear in the lingual epithelium just before fungiform taste buds form (Farbman, 1965; Farbman and Mbiene, 1991; Whitehead and Kachele, 1994; Witt and Reutter, 1996).

[1] To whom correspondence shoould be addressed. Fax (859) 323-2094. E-mail: rkrimm@pop.uky.edu.

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Once innervated, both papillae and taste buds are dependent on innervation for their continued growth and maintenance (Hosley *et al.,* 1987; Nagato *et al.,* 1995). Therefore, a lack of gustatory innervation is reflected by a quantifiable loss of taste buds and papillae, making the taste system ideal for studies of signaling factors that influence sensory organ development.

Two growth factors essential for taste system development are the neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4). Similar to other neurotrophin family members, i.e., nerve growth factor (NGF) and neurotrophin-3 (NT3), the expression of BDNF and NT4 by target tissues is thought to support the development and maintenance of specific neuronal populations. Consistent with this role in the taste system, BDNF mRNA is expressed in cells of the developing gustatory epithelium and adult taste buds (Nosrat *et al.,* 1996; Nosrat and Olson, 1995). In addition, $BDNF^{-/-}$ mice have a severe reduction of gustatory neurons in the geniculate (48%) and nodose-petrosal (43–64%) ganglia (Conover *et al.,* 1995; Ernfors *et al.,* 1994; Jones *et al.,* 1994; Liu *et al.,* 1995). Mice that lack NT4 also have a severe reduction of neurons in the geniculate (50%) and nodose-petrosal (56%) ganglia. This loss was additive in $BDNF^{-/-}$ and $NT4^{-/-}$ double knockout mice (Conover *et al.,* 1995; Liu *et al.,* 1995) and in mice that lack the BDNF and NT4 tyrosine kinase receptor, trkB (Fritzsch *et al.,* 1997). Coincident with this neuronal loss, taste buds and gustatory papillae are also absent in $BDNF^{-/-}$ (Mistretta *et al.,* 1999; Nosrat *et al.,* 1997; Oakley *et al.,* 1998; Zhang *et al.,* 1997) and $NT4^{-/-}$ mice (Liebl *et al.,* 1999). In contrast to gustatory innervation, somatosensory innervation of lingual structures (e.g., filiform papillae) is provided by neurons of the trigeminal ganglia and is lost in $NT3^{-/-}$ mice (Farinas *et al.,* 1994; Nosrat *et al.,* 1997; Wilkinson *et al.,* 1996). Thus, BDNF and NT4 selectively sustain both gustatory neurons and the taste buds they innervate, whereas NT3 specifically maintains somatosensory innervation to the rest of the tongue.

In addition to their role in neuron survival, the spatial pattern of neurotrophin expression may be important for target selection within the developing tongue epithelia, allowing gustatory and somatosensory innervation to distinguish their respective targets during axonal guidance (Nosrat and Olson, 1998; Ringstedt *et al.,* 1999). To examine this issue, the taste system in mice that overexpress BDNF under control of the nestin promoter was studied (Ringstedt *et al.,* 1999). The nestin promotor directed expression of BDNF to neuroepithelial stem cells of the CNS and PNS and to developing muscle (Lendahl *et al.,* 1990). Nestin-BDNF mice die just prior to or after birth, precluding study of the developing postnatal and adult taste system. In embryonic nestin-BDNF mice, gustatory axons failed to invade their taste bud targets and stalled in the tongue muscle where BDNF levels were high (Ringstedt *et al.,* 1999). This finding suggested that the level of BDNF within the tongue and/or within gustatory neurons is important for target invasion. However, since innervating fibers remained in the subepithelial musculature, this study did not provide direct evidence that distribution of BDNF within the lingual epithelia influences the ability of gustatory neurons to select taste buds, over adjacent epithelia, as their appropriate target. To test the role of BDNF and NT4 made by the epithelium, we examined the taste system in mice that overexpress BDNF or NT-4 in basal epithelial cells under the control of a keratin-14 promoter. Analysis of innervation in K14-BDNF transgenics showed clear disruption of neuronal targeting to the taste epithelium. These studies also extend the findings of Ringstedt *et al.* (1999) by providing quantification of the number and size of geniculate ganglion neurons, fungiform papillae, and taste buds following neurotrophin overexpression, and by comparing the effects of K14-BDNF overexpression with overexpression of the other ligand for trkB, NT4.

MATERIALS AND METHODS

Animals

The K14-BDNF-OE transgenic mice used in this study are thoroughly described in LeMaster *et al.* (1999). K14-NT4-OE mice were isolated by microinjecting a K14 promoter-driven NT4 cDNA (a gift from P. Ernfors, Karolinska Institute, Sweden) into pronuclei of embryos obtained from B6 x C3H F1 hybrid females (Harlan Sprague Dawley, Indianapolis, IN) as described previously (Albers *et al.,* 1994; LeMaster *et al.,* 1999). Founder and F1 generations were analyzed using either Southern blotting, slot blotting, or reverse transcriptase polymerase chain reaction (RT-PCR) techniques to identify transgenic lines, verify transgene integration, and estimate copy number. Animals were cared for and used in accordance with guidelines of the *U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and NIH Guide for the Care and Use of Laboratory Animals.*

In Situ Hybridization

In situ hybridization to detect BDNF mRNA was done using ^{35}S-labeled probes as previously described (Albers *et al.,* 1994). Tongues from 1-day-old mice were frozen on dry ice, cut into 15-μm sections, and thaw mounted onto Superfrost slides. Sections were brought to room temperature, immersion fixed for 15 min in 4% paraformaldehyde in phosphate buffered saline (PBS), and then washed in PBS, PBS with 0.2% glycine, and 0.25% acetic anhydride in 0.1 M TEA, pH 8.0. Sections were dehydrated, air dried, and incubated with probe hybridization solution (Amresco, Solon, OH) containing 1×10^6 cpm per 50 μl. A glass coverslip was placed over the probe and secured to the slide by applying mounting media around the cover glass. Slides were incubated overnight on a hot plate at 60°C and then dipped in NTB2 photographic emulsion, exposed 1–2 weeks, developed, and counterstained with hematoxylin and eosin. Sense transcript controls processed in parallel showed no specific hybridization.

Geniculate Ganglion Cell Counts and Diameters

Transgenic and littermate control mice were deeply anesthetized using avertin and perfused with 4% paraformaldehyde in phosphate buffer. Ganglia were removed, immersion fixed 2–4 h,

dehydrated, and embedded in paraffin. Neuron counts in adult geniculate ganglia were obtained using previously described methods (Coggeshall *et al.*, 1990; Goodness *et al.*, 1997). Briefly, ganglia were serially sectioned at 5 μm, nissl-stained with cresyl violet, and neurons with nucleoli present in six equally spaced sections were counted at $\times$400. The neuron number was multiplied by the total number of sections to obtain a total neuron count. To compensate for neurons with two or more nucleoli, profiles of randomly selected neurons were reconstructed and the number of nucleoli per 100 neurons determined. This ratio was multiplied by the total neurons counted to obtain the total number of neurons per ganglion. Neuron diameters were calculated from measures of somal areas of 100 neurons sampled from the middle section of each ganglia.

Papillae Number

Paraformaldehyde-fixed tongues were washed in phosphate buffer, the muscle underneath the epithelial surface removed, and the surface stained with a concentrated solution of methylene blue. The epithelial sheet was placed on a glass slide and coverslipped using glycerol. Maps of the tongue surface were traced to localize fungiform papilla, the intermolar eminence, and borders of the tongue using a drawing tube attached to an Olympus microscope (Olympus Corp., Lake Success, NY).

Measure of Taste Bud Number and Size

Paraformaldehyde-fixed tissue blocks containing the posterior tongue were dissected to include the circumvallate papilla and some surrounding tissue. The anterior tongue was hemisected at the midline and half examined for fungiform taste bud number and size. Tissues were paraffin embedded, serially sectioned at 8 μm, and stained with hematoxylin and eosin. For circumvallate and nasoincisive papillae, sections were examined and taste buds containing pores identified and recorded. The number of fungiform papillae per tongue (obtained from tongue maps), was multiplied by the mean number of taste buds/papilla to estimate the total number of fungiform taste buds. To measure the size of fungiform taste buds, their borders were outlined in each section and the area was measured using NIH Image software. To achieve consistent measures across taste buds, borders were drawn to include peripheral cells of the taste bud (Whitehead *et al.*, 1985). The area values were multiplied by section thickness and summed across all sections containing a taste bud to estimate total taste bud volume. Taste bud diameters were calculated from the taste bud area of the middle section of each taste bud. Diameters of fungiform papilla were determined from measures across their widest point.

Immunohistochemistry

To detect the distribution of keratin-14 protein expression, paraffin-embedded tissue was sectioned at 10 μm, mounted on slides, dewaxed, rehydrated, and treated with proteinase K. Sections were incubated with a monospecific polyclonal keratin-14 antibody (Stoler *et al.*, 1988) overnight at room temperature, washed, incubated 1 h in a 1:500 dilution of biotinylated goat anti-rabbit secondary, and then incubated with a strep-avidin complex (Vector Laboratories, Burlingame, CA). Antibody binding was visualized using a nickel cobalt-enhanced diaminobenzidene reaction. Tongue innervation in P0 mice was detected by immunolabeling tissue from the midregion of unfixed tongues that were embedded in OCT and serially sectioned on a cryostat at 20 μm. Slides were placed in acetone for 10 min at 20°C, rinsed in PBS, incubated with anti-NF150 (Chemicon, Temecula, CA) overnight at room temperature, washed, and incubated in goat anti-rabbit Cy2 for 1 h. Slides were washed, dried, cleared through alcohols and HemoDe (Fisher Scientific, Springfield, NJ), coverslipped with DPX mounting media (BDH Laboratory Supplies, Poole, UK), and viewed using a Leica confocal microscope located in the University of Kentucky Imaging Center.

Nerve Labeling Using 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)

Embryonic mice were obtained from mouse breedings set up just prior to the 8-h dark period. The following morning males were removed from the cages. This day was designated embryonic day 0.5 (E0.5). E18.5 embryos were deeply anesthetized, perfused and postfixed with 4% phosphate buffered paraformaldehyde. The next day the brain and trigeminal ganglia were removed and DiI crystals (Molecular Probes, Eugene, OR) placed on the facial nerve followed by a drop of 100% EtOH. Embryos were placed between two buffer-soaked towels for 2 h, returned to 4% paraformaldehyde, and placed at 37°C for 8–12 weeks. After incubation, the heads or lower jaws were sectioned at 70–100 μm on a vibratome. Sections were coverslipped with PBS and immediately viewed using a Leica confocal microscope.

Statistical Analysis of Data

Analysis of variance (ANOVA) was used to compare geniculate ganglion cell number and size, numbers of fungiform papillae and taste buds in adult mice, fungiform papillae diameter, and taste bud volume and diameter across genotypes. Individual means were compared using the Fischer's least-significant difference (LSD) procedure. Student's *t* tests were used to compare the number of circumvallate taste buds, nasoincisal taste buds, number of fungiform papillae, and surface area in newborn mice. Although the alpha level was set at $P < 0.05$, the actual *P* values are reported.

RESULTS

K14-Driven Transgene Expression Occurs across the Tongue Basal Epithelium

This study examined the role of BDNF and NT4 neurotrophins in taste system development and innervation using transgenic mice that overexpress these growth factors in basal epithelial cells of the tongue. Transgene expression was regulated using enhancer and promoter elements from the human K14 keratin gene (Fig. 1A). This well-characterized promoter system drives high levels of mRNA expression in K14-expressing epithelium such as the skin and tongue in a proper temporal and spatial manner (Byrne *et al.*, 1994; LeMaster *et al.*, 1999; Wang *et al.*, 1997). K14 is expressed across interpapillae basal epithelial cells and basal cells of filiform, fungiform, and circumvallate papillae (Fig. 1B) (Takami *et al.*, 1995). Robust K14 protein expression in rat tongue epithelium has been reported to occur by embryonic day 15 (E15) (Shuler and Schwartz, 1986). Thus, transgene-driven BDNF expression overlaps with expres-

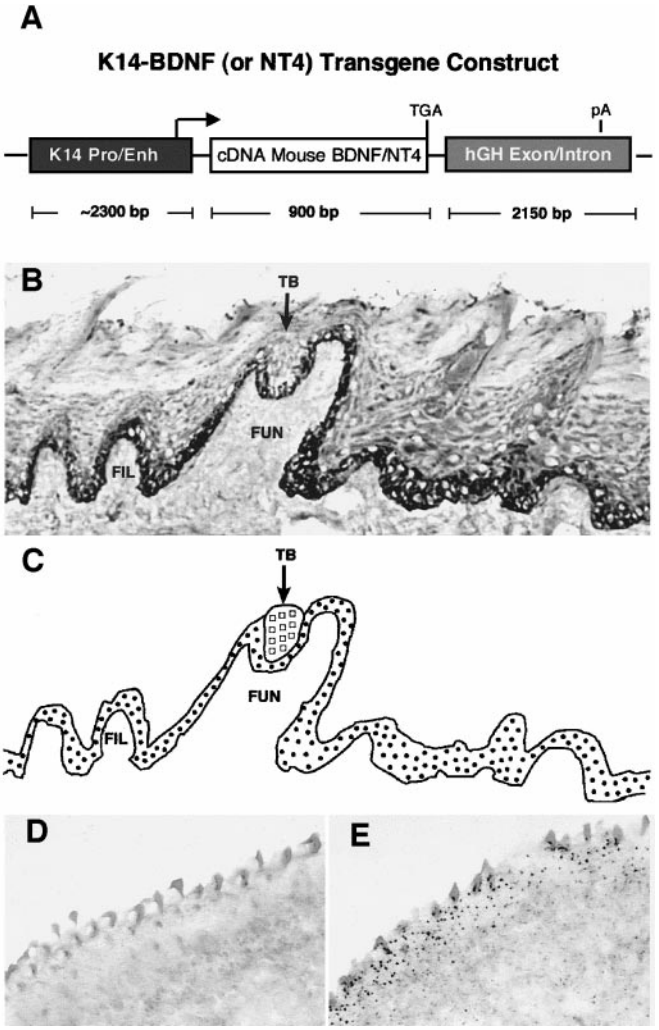

FIG. 1. The K14 keratin promoter drives expression of BDNF and NT4 in tongue epithelium. (A) Expression of BDNF or NT4 cDNA was driven by 2.3 kbp of promoter and enhancer sequences of the human K14 gene (LeMaster *et al.*, 1999; Wang *et al.*, 1997). Arrow indicates transcriptional start site. The human growth hormone (HGH) sequence provides intron/exon splice sites and a polyA addition signal. (B) Anti-K14 immunolabeling of tongue sections shows the location of K14 containing cells along the basal epithelial layer in both fungiform (FUN) and filiform (FIL) papillae. K14 is expressed in extragemmal cells that surround fungiform taste buds, but not in the taste bud itself. (C) The cellular expression pattern of endogenous BDNF (open squares) and transgenic BDNF or NT4 (filled circles) in the adult tongue (adapted from Nosrat *et al.*, 1996). (D, E) *In situ* hybridization using a ^{35}S-labeled antisense probe to mouse BDNF mRNA. Sections were examined after 2 weeks of exposure to emulsion and confirmed K14-BDNF transgene expression in epithelial cells of the tongue of P0 transgenic mice (E). No specific signal was detected in sections from control tongue at exposure times examined (D).

sion of endogenous BDNF, which in rat appears in epithelium of developing fungiform and circumvallate papillae by E15 (Nosrat and Olson, 1995). By postnatal day 1, endogenous BDNF mRNA is restricted to epithelial cells of developing taste buds, and is absent from nongustatory epithelium (Nosrat *et al.*, 1996). This pattern of restricted expression does not occur in BDNF-OE mice, in which, relative to control mice, high BDNF mRNA levels are maintained throughout the epithelium (Figs. 1D and 1E). This expression parallels ELISA measurements of tongue extracts that showed undetectable levels of BDNF peptide in controls and high levels in BDNF-OEs (LeMaster *et al.*, 1999). Thus, in postnatal and adult BDNF-OE and NT4-OE animals, K14-driven transgene overexpression does not overlap with the endogenous pattern of BDNF expression.

Survival of Geniculate Ganglion Neurons Is Increased in BDNF-OE and NT4-OE Transgenic Mice

The geniculate ganglion provides taste afferents that innervate fungiform papillae at the front and midregion of the tongue. BDNF and NT4 are important for geniculate neuron survival, as demonstrated by the 48 and 50% loss of these neurons in BDNF$^{-/-}$ and NT4$^{-/-}$ knockout mice, respectively (Liu *et al.*, 1995). The opposite effect was found in overexpresser transgenics, where the geniculate ganglion of both BDNF-OE and NT4-OE mice appeared substantially larger than ganglia of control mice (Fig. 2A). Neuronal cell counts to verify this increase (Fig. 2B) showed BDNF-OE ganglia had 93% more neurons relative to controls (1684 $\pm$ 150 vs 873 $\pm$ 44.7), whereas NT4-OE mice (2097 $\pm$ 118) had a 140% increase.

In addition to increasing cell number, excess BDNF and NT4 resulted in larger geniculate ganglion neurons (Figs. 2C–2E). Cell soma diameters plotted as a frequency histogram showed the mean diameter of geniculate neurons of BDNF-OE mice (14.5 $\pm$ 0.8 μm) and NT4-OE mice (13.5 $\pm$ 0.73 μm) were significantly greater than that of control neurons (10.1 $\pm$ 0.4 μm; Fig. 2F).

Although More Geniculate Neurons Are Present, the Numbers of Fungiform Papillae and Taste Buds Are Decreased

Despite having more neurons in the geniculate ganglia, the number of fungiform papillae, which depend on these neurons for postnatal survival, was substantially reduced in BDNF-OE and NT4-OE mice compared to that of age-matched controls (Figs. 3A–3C). Maps of the distribution of fungiform papillae showed absence of papillae in the midregion of most BDNF-OE (Fig. 3H) and NT4-OE (Fig. 3I) tongues. The number of papillae on the dorsal and ventral tip of the tongue was also reduced in transgenic animals. Interestingly, fungiform papillae in the caudal tongue around the intermolar eminence (IM) were not reduced in transgenics, although their location was abnormal. This was particularly evident on NT4-OE tongues, where fungiform papillae extended caudally around the IM.

Reflecting the loss of fungiform papillae, adult BDNF-OE

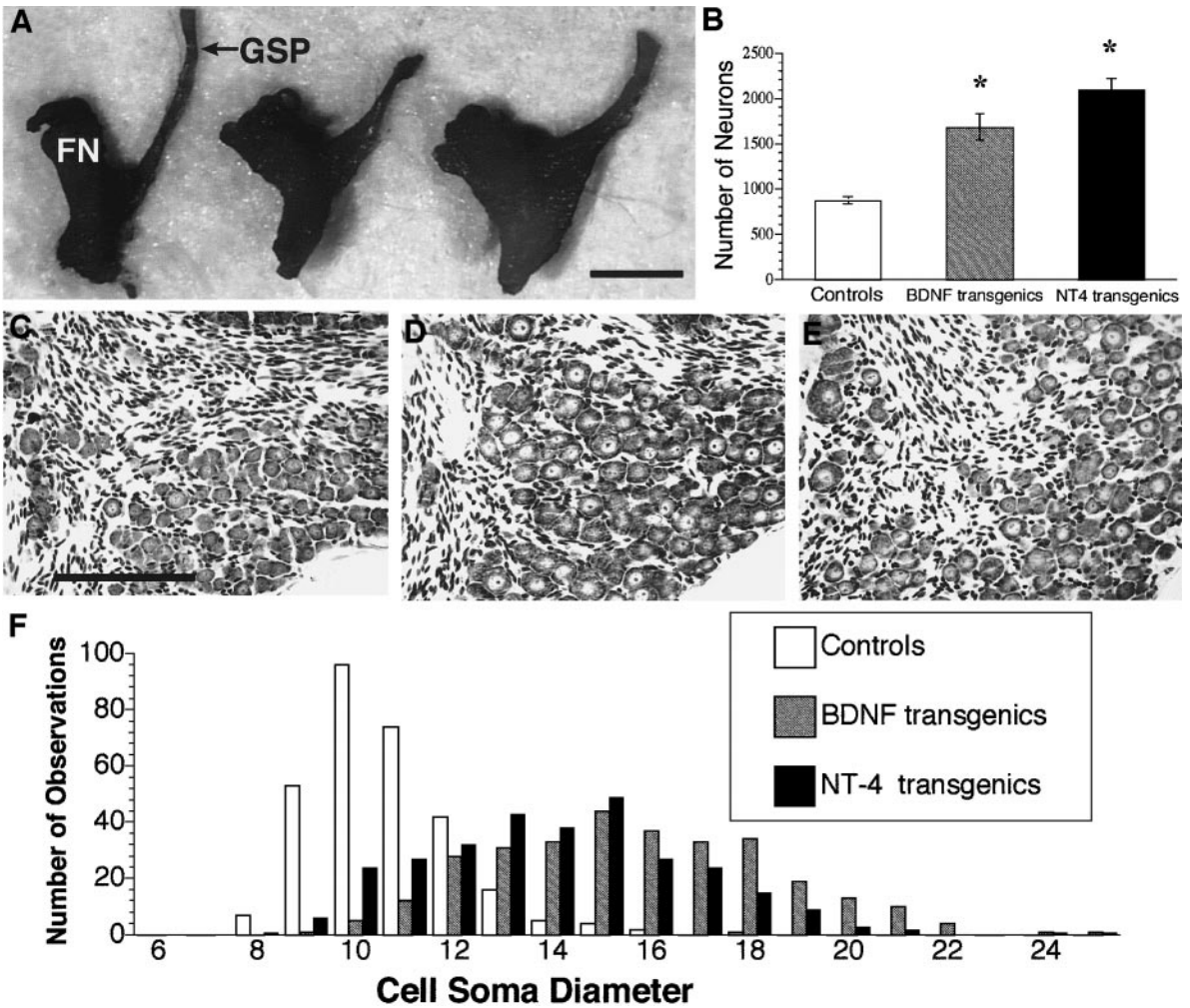

FIG. 2. Number and size distribution of geniculate ganglion neurons in transgenic and control mice. (A) Cell bodies of the eosin-stained geniculate ganglion are located at the junction of the facial nerve (FN) and the greater superficial petrosal nerve (GSP). The geniculate ganglion of BDNF-OE (middle) and NT4-OE (right) are larger than that of control (left). (B) The number of geniculate ganglion neurons in control (n = 4; 873 ± 44.7), BDNF-OE (n = 6; 1684 ± 150), and NT4-OE (n = 3; 2097 ± 118) mice. Asterisks denote statistically significant differences from controls ($P = 0.001$, BDNF-OE; $P = 0.0002$, NT4-OE). (C–E) Cell bodies of geniculate ganglion neurons appear larger in nissl-stained sections of BDNF-OE (D) and NT4-OE (E) mice compared with controls (C). (F) Size distribution analysis of geniculate ganglion cell diameters in BDNF-OE and NT4-OE mice showed transgenic neurons had, on average, larger somas than neurons of control mice ($P = 0.004$; $P = 0.02$, respectively). Scale bar = 1 mm in A. Scale bar = 100 μm in C and applies to C, D, and E.

and NT4-OE mice also had fewer fungiform taste buds relative to control mouse values (control, 93.7 ± 4.9; BDNF-OE, 26.2 ± 1.3; NT4-OE, 29.3 ± 2.3; see Table 1). In addition to their location in fungiform papillae on the anterior tongue, taste buds are also in circumvallate and foliate papillae on the back of the tongue, the hard and soft palate, and the epiglottis (Miller and Spangler, 1982). Taste bud loss in overexpressers was specific to the front of the tongue, because counts of taste buds in circumvallate and nasoincisive papilla of the hard palate of BDNF-OE mice were similar to control values (Table 1). Thus, the loss of taste buds caused by neurotrophin overexpression was specific to fungiform papillae.

Fungiform Papillae Are Formed in Transgenic Mice during Embryogenesis, but Lost Postnatally

The loss of fungiform papillae and taste buds in adult transgenics may have been caused by degeneration of formed papillae and taste buds, or alternatively, incomplete papillae and taste bud formation during development. We tested these possibilities in the BDNF-OE animals by

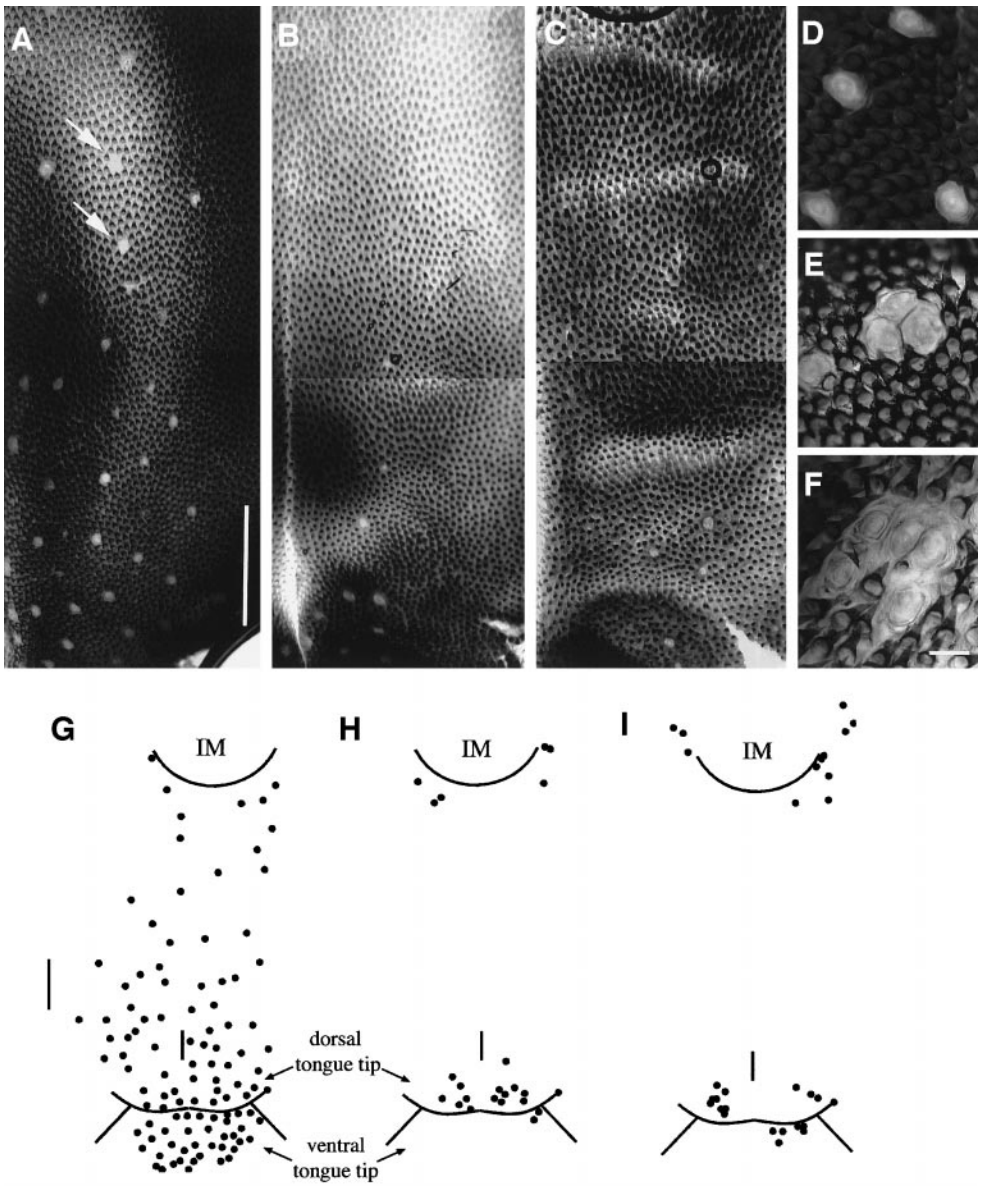

FIG. 3. Photomicrographs of the tongue surface and maps of tongue papillae locations in control (A, D, G), BDNF-OE (B, H), and NT4-OE (C, E, F, I) mice. (A–C) Dorsal tongue tip and the midregion of the left half of a control (A), BDNF (B), and NT4-OE (C) mouse. Arrows in A denote papillae. (D–F) High magnification photomicrographs of fungiform papillae on the tip (D, E) and around the IM (F) of a control (D) and NT4-OE (E, F) tongue. (G–I) Tongue maps illustrate typical papillae distributions in control (G), BDNF-OE (H), and NT4-OE (I) animals. Papilla loss was greatest in the tongue midregion, with sparing around the intermolar eminence (IM). While the fungiform papillae of control mice are typically spaced apart (D), the papillae of transgenic mice, particularly NT4-OE mice, frequently occur in clumps of two or more (E, F). Scale bar in A = 1 mm and applies to A, B, and C. Scale bar in F = 100 μm and applies to D, E, and F. Scale bar in G = 1 mm and applies to G, H, and I.

counting the number of fungiform papillae on the day of birth. No significant difference in papilla number was found between control (83 ± 1.87) and BDNF-OE (71.3 ± 12.7) mice (Figs. 4A and 4B). However, papillae on the midregion of P0 tongues, which are destined to disappear in adult transgenic mice, occupied a smaller surface area in BDNF-OE mice (2369 ± 62 μm^2) than in control mice (3930 ± 355 μm^2). To examine nerve innervation to these smaller midregion papillae, tongues from newborn BDNF-OE (Figs. 5D–5F) and control (Figs. 5A–5C) mice were immunolabeled using an anti-neurofilament-M antibody. A substantial reduction in innervation to fungiform

TABLE 1
Quantification of Taste Bud Number and Size

	Control (n = 4)	BDNF-OE (n = 3)	NT4-OE (n = 3)
Number of taste buds			
Fungiform	93 ± 5	26 ± 1*	29 ± 2*
Circumvallate	185 ± 10	190 ± 17	N.D.
Incisal papillae	10 ± 2	10 ± 1	N.D.
Size of taste buds			
Volume (μm^3)	$2.1 \times 10^4 \pm 0.1$	$2.0 \times 10^4 \pm 0.1$	$1.9 \times 10^4 \pm 0.2$
Taste bud diameter (μm)	34.5 ± 1.7	34.7 ± 1.6	32.9 ± 0.1
Papillae diameter (μm)	77.8 ± 7.8	70.8 ± 2.5	73.4 ± 5.1

Note. The number of taste buds associated with fungiform papillae of BDNF-OE and NT4-OE mice were reduced relative to controls. However, no difference in taste bud number in incisal and circumvallate papilla of BDNF-OE was measured, suggesting a fungiform specific effect. The volume and/or diameter of fungiform taste buds and papillae remaining in BDNF-OE and NT4-OE mice were similar to control values. Values are expressed as standard errors of the mean. N.D., not determined.

* Significantly different from control measures.

papillae in transgenic mice relative to controls was evident (compare Fig. 5E, transgenic and Fig. 5B, control). Though some innervation to papillae remained in BDNF-OE tongues, this innervation may be somatosensory (NT3-dependent) and not gustatory.

Gustatory Neurons Innervate Inappropriate Targets in Transgenic Mice

Although BDNF-OE and NT4-OE transgenic mice had significantly more geniculate ganglion neurons, fungiform papillae targets normally innervated by these afferents were lost postnatally. To identify the projection patterns of these excess neurons and to understand why taste papillae were lost, the chorda tympani nerve, which innervates fungiform taste buds, was labeled in E18.5 embryos with the fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). Innervation to fungiform papillae in control animals was well established, discrete, and spatially patterned (Figs. 6A and 6C). NT4-OE (Figs. 6B, 6E, 6G, and 6H) and BDNF-OE (Figs. 6D, 6F, and 6I) embryos had DiI-labeling in large nerve bundles projecting caudal to rostral along the base of the tongue. In both BDNF-OE and NT4-OE mice fewer branches exited the large primary nerve in the midregion of the tongue, and virtually no fungiform papillae were innervated in this region. However, in BDNF-OE mice some papillae in the tongue tip and caudal tongue were innervated. In both BDNF-OE and NT4-OE mice, some nerve bundles ended in tight complexes of neural fibers that remained below the tongue epithelium (Fig. 6G). In NT4-OE mice, large bundles within the tongue tip expanded as they neared the epithelial surface and innervated wide regions of lingual epithelium (Fig. 6H) or split into branches that traveled parallel to the tongue surface (Fig. 6E). In BDNF-OE mice, we also observed chorda tympani nerve bundles that projected toward individual fungiform papillae, but also aberrantly innervated adjacent filiform papillae (Figs. 6D and 6I). Also, in BDNF-OE mice small branches were observed innervating regions of lingual epithelium where no fungiform papillae were evident (Fig. 6F). To summarize, at E18.5 many DiI-labeled fibers approached but did not penetrate the transgenic epithelium. Aberrant innervation to filiform papillae and reduced innervation to fungiform papillae was evident for both transgenic lines, although some differences in the pattern of this innervation exist.

Clusters of Fungiform Papillae Are Formed and Some Papillae Develop Two Taste Buds in Transgenic Mice

Although the fungiform papillae and taste buds that remained in adult BDNF-OE and NT4-OE mice were normal in size, some unusual morphology was observed. For example, papillae in control mice (n = 3) contained one taste bud, whereas 4% of BDNF-OE papillae and 1% of NT4-OE papillae contained two taste buds (Figs. 7B, 7C, and 7E). Most interesting was the distribution of the remaining papillae on the tongues of transgenic mice. Many papillae occurred in pairs (Figs. 7D and 7E) or clusters of three or more (Figs. 3E and 3F; Figs. 7F, 7G, and 7H), particularly in NT4-OE mice. In BDNF-OE mice, 10% of papillae were found in this arrangement, whereas NT4-OE mice had 63% of papillae immediately adjacent to another papilla. Papillae pairs and clusters were not found on tongues of the three control mice examined.

DISCUSSION

Role of Neurotrophins and Innervation in Taste Bud Development

Geniculate ganglion neurons are dependent on BDNF and NT4 for their survival (Ernfors *et al.*, 1994; Liu *et al.*, 1995). Consistent with their role as survival factors, we found that

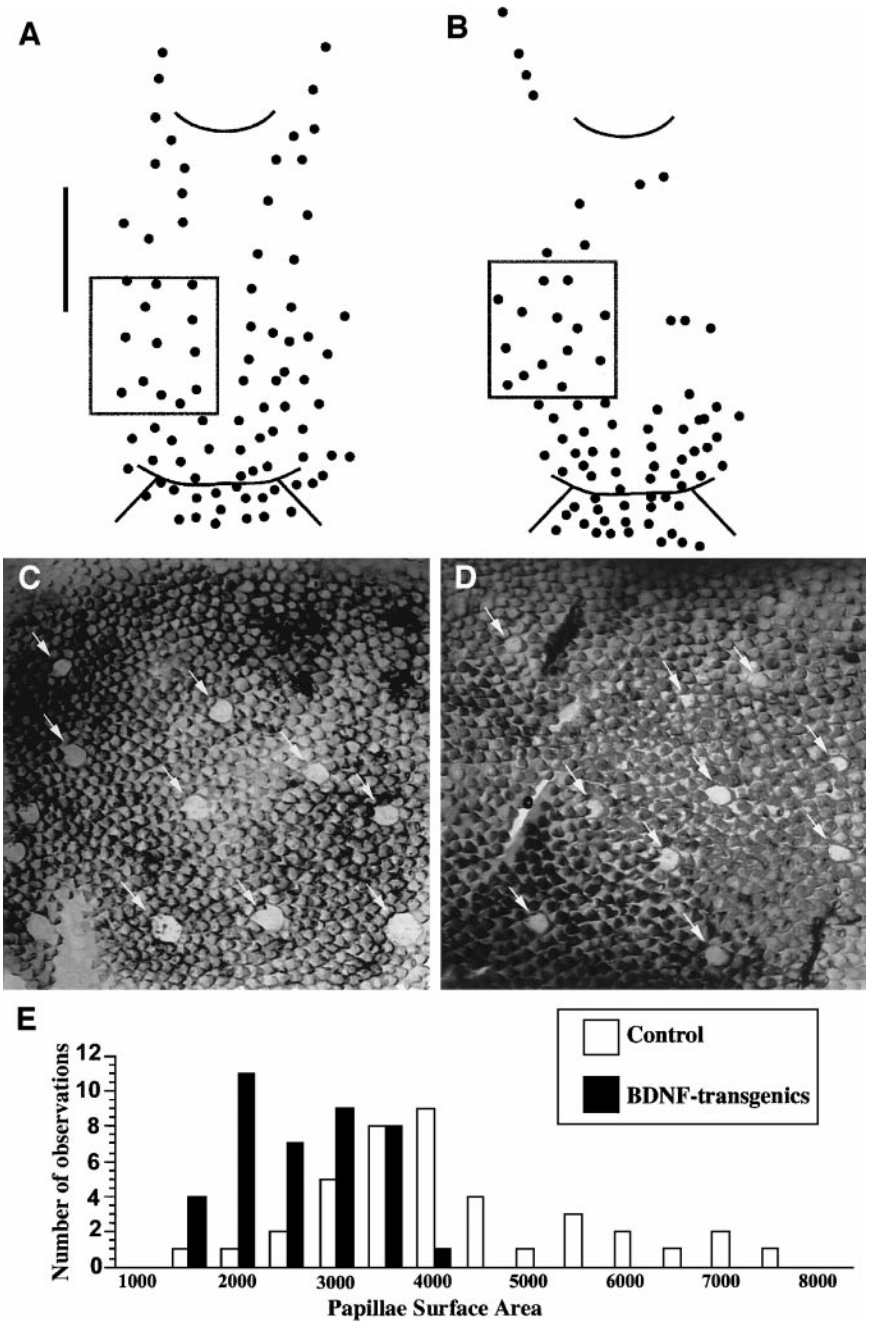

FIG. 4. Papillae maps and size in P0 control and BDNF-OE mice. (A, B) At P0, no significant difference in the number of papillae between BDNF-OE and control mice was measured ($P = 0.16$). (C, D) Photomicrographs of the tongue midregion surface (indicated by the squares in A and B) show BDNF-OE fungiform papillae are smaller than control papillae (C). (E) Surface areas of papillae in the tongue midregion plotted as a function of the number of papillae per area show transgenic papillae were atrophied relative to controls ($P = 0.006$). Bar = 1 mm.

overexpression of these growth factors caused a 93–140% increase in the number of geniculate neurons. In these same animals, we observed a considerable loss of fungiform papillae and taste buds on the tongues of BDNF-OE mice and NT4-OE mice, which appears to be the result of a lack of gustatory innervation. In mammals, gustatory nerve fibers are not required for taste papillae formation (Mbiene *et al.*, 1997), but are crucial for their maintenance and

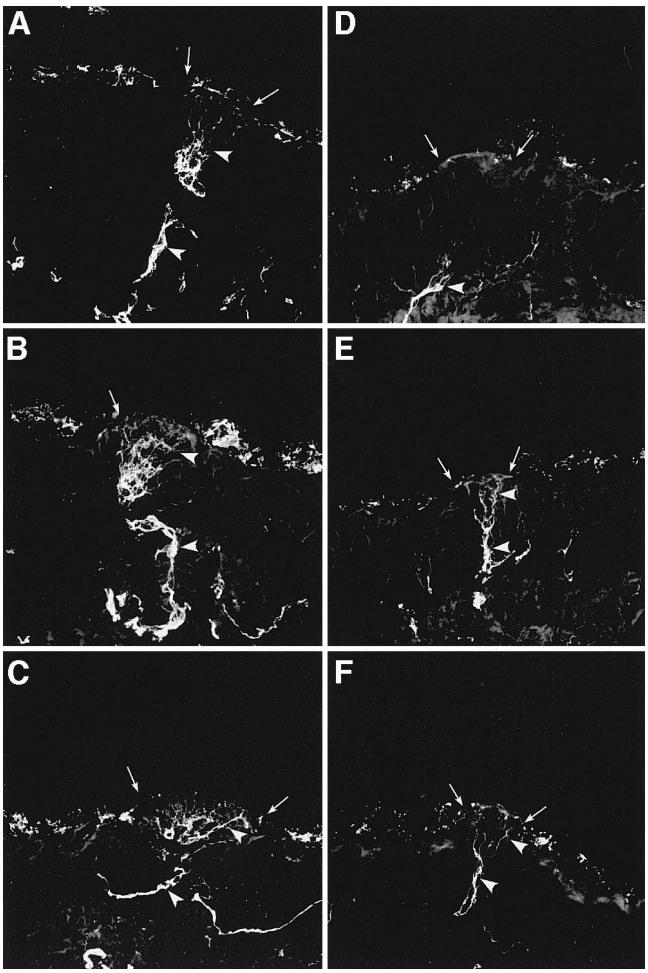

FIG. 5. Innervation of P0 transgenic and control mouse tongue epithelium was detected by anti-neurofilament-M immunolabeling. Comparison of serial sections through fungiform papillae in the midregion of control mice (A–C) and BDNF-OE mice (D–F) showed reduced innervation in transgenic samples. Arrows indicate papilla edge, arrowheads point to neurofilament-M labeled fibers. Bar = 100 μm.

growth (Hosley *et al.*, 1987; Morris-Wiman *et al.*, 1999; Nagato *et al.*, 1995; Oakley *et al.*, 1990). Thus, even though excess taste neurons were generated in BDNF-OE and NT4-OE mice, the failure of these afferents to innervate fungiform papillae resulted in postnatal papillae loss. The few papillae that remained in the adult were most likely maintained by either the few chorda tympani axons that successfully reached the taste bud or by innervation from trigeminal afferents (Kinnman and Aldskogius, 1991).

In addition to neuronal influences on development and maintenance of taste papillae and buds, our findings point out the importance of local cell–cell interactions (Barlow *et al.*, 1996; Barlow and Northcutt, 1997). Even though gustatory nerve fibers innervated nongustatory filiform papillae in BDNF-OE and NT4-OE mice, taste buds did not form in these areas. This result confirms the multifactorial nature of taste bud formation and the importance of the taste papillae epithelium and local signaling factors.

BDNF and NT4 Influence Target Selection in the Developing Taste System

Neurotrophin expression by the tongue epithelium has been proposed to direct targeting of innervating sensory neurons (Nosrat and Olson, 1998; Ringstedt *et al.*, 1999). This possibility has been examined using transgenic mice that ectopically overexpressed BDNF under control of the nestin promotor, which directed expression of BDNF to developing neurons and muscle. Gustatory axons of nestin-BDNF mice failed to invade their epithelial taste bud targets and remained in the musculature at the base of the tongue (Ringstedt *et al.*, 1999). This aberrant targeting was likely the result of high levels of BDNF expression within the tongue, although it could also reflect disrupted target recognition caused by the enhanced expression of BDNF in the taste neurons themselves. A similar failure of target innervation is found in animals that overexpress NGF in sympathetic neurons under control of the dopamine β-hydroxylase promoter. In these mice sympathetic axons grow toward their peripheral targets but fail to innervate them (Hoyle *et al.*, 1993). In another example, the nestin promoter was used to overexpress NT3 in developing muscle and neurons and caused a loss of limb proprioceptive endings (Ringstedt *et al.*, 1997). In contrast, restricted overexpression of NT3 to muscle, using the myogenin promoter, resulted in an increase in proprioceptive endings (Wright *et al.*, 1997). Thus, neurotrophin overexpression in neurons can inhibit target invasion, perhaps by preventing neurons from recognizing external neurotrophin gradients (Hoyle *et al.*, 1993).

In the present study, overexpression of BDNF or NT4 in nontaste epithelia prevented gustatory neurons from innervating appropriate taste papillae targets and instead, caused abnormal innervation of tongue regions that do not typically receive gustatory input, including filiform papillae. This misdirection of taste afferents into nontaste papillae suggests the spatial distribution of BDNF and NT4 within lingual epithelia is important for appropriate target selection by chorda tympani axons. At present we cannot discriminate between the BDNF- and NT4-induced alteration of axon targeting because both transgenic lines exhibited misdirection of afferents and subsequent loss of fungiform papillae and taste buds. However, the similarity of the effects is not unexpected, because both ligands bind the trkB receptor tyrosine kinase with equal affinity (Klein *et al.*, 1991, 1992). Furthermore, knocking NT4 into the BDNF locus rescues geniculate ganglion cells in BDNF-deficient mice (Fan *et al.*, 2000), indicating that NT4 is capable of assuming the function of BDNF for developing gustatory ganglia. A more detailed analysis of BDNF-OE and NT4-OE mice across development to evaluate potential

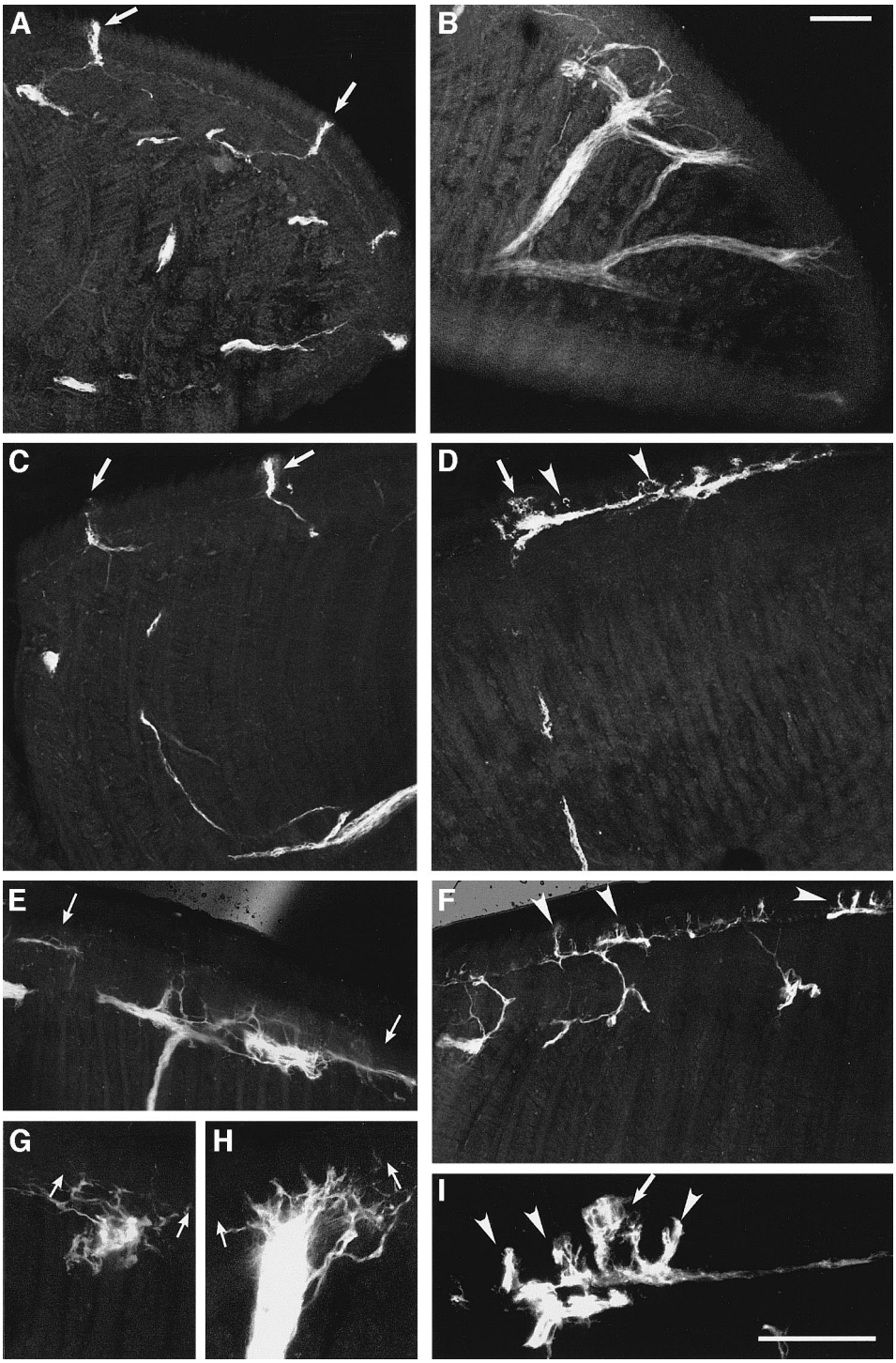

FIG. 6. Innervation of the tongue by taste afferents demonstrated by DiI-labeling of the facial nerve of E18.5 embryos. Sagittal sections of tongue in control (A, C), NT4-OE (B, E, G, H), and BDNF-OE (D, F, I) mice. In control mice, nerve bundles were present in tongue muscle and innervation to the epithelium occurred specifically to fungiform papillae (arrows in A, C). Nerve bundles in muscle of NT4-OE and BDNF-OE samples were larger than those in control samples (compare A to B) and enlarged as they approached the epithelium (B, H). Arrows indicate the dermal–epithelial border in E, G, and H. Some bundles stayed below the epithelium, but ran parallel to the tongue surface for long distances (E). Other bundles terminated in tight complexes near the epithelium (G). Taste afferent innervation to the tongue surface of transgenics was not limited to fungiform papillae (arrows in D and I) but was also found within nontaste filiform papillae (arrowheads in D, F, and I). Scale bar = 100 μm in B and applies to A–F. Scale bar = 100 μm in I and applies to G–I.

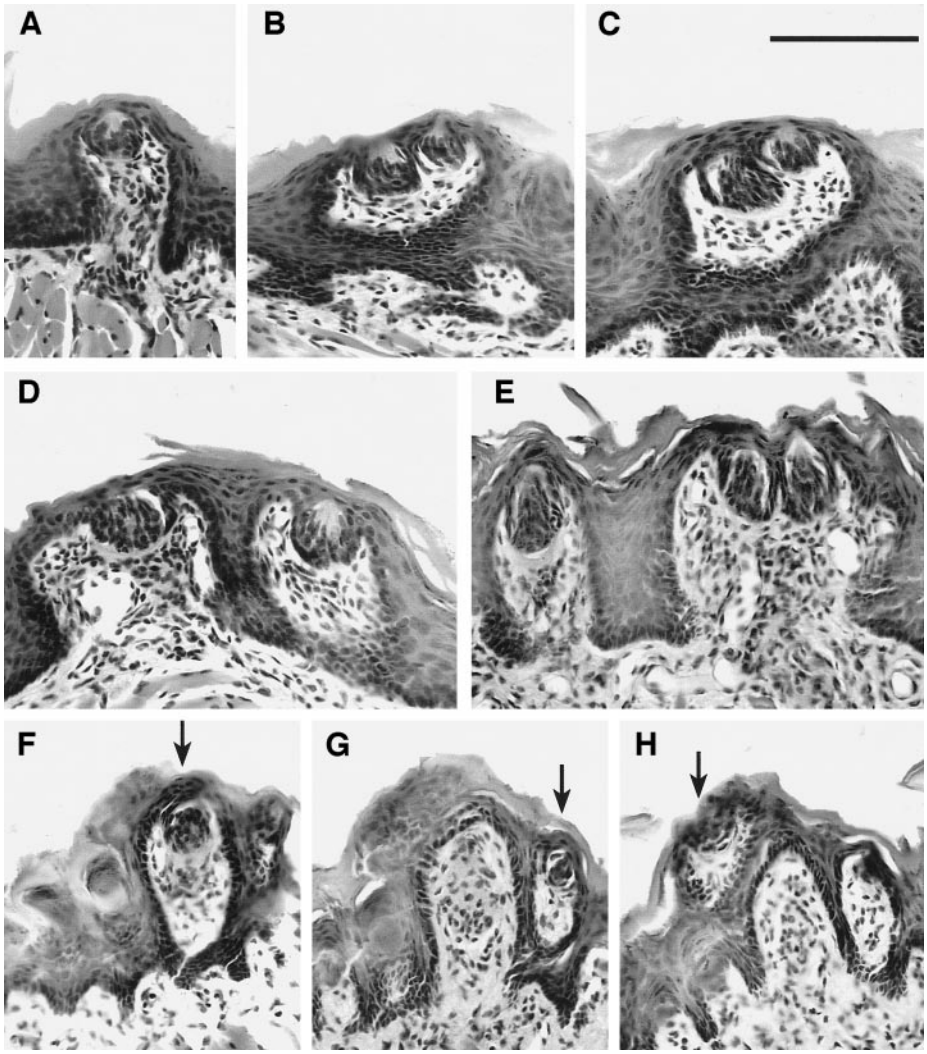

FIG. 7. Comparison of fungiform papillae from control (A), BDNF-OE (B, C, D), and NT4-OE mice (E, F, G, H). Fungiform papilla of control animals had one taste bud (A), while some papillae in transgenic lines contained two taste buds (B, C, E). Many fungiform papillae in transgenic mice also occurred in pairs (D, E). Serial sections (F, G, H) show a clump of three papillae in a NT4-OE mouse. Scale bar = 100 μm.

differences in the timing and/or pattern of chorda tympani innervation and to define the subpopulations of geniculate ganglion neurons affected in transgenic mice is required.

How chorda tympani axons become misdirected to inappropriate targets in BDNF-OE and NT4-OE mice is unclear. One possible mechanism is that BDNF and NT4 act as chemotropic factors for these afferents. *In vitro* studies using growth cones of Xenopus spinal neurons and mammalian DRG neurons have shown that neurotrophin gradients can alter the course of growing neurites (Ming *et al.*, 1997, 1999; Paves and Saarma, 1997). These gradient responses are factor specific in that growth cones of NGF-dependent DRG neurons turn toward a source of NGF, whereas BDNF causes collapse of these same neurons (Paves and Saarma, 1997). Similarly, growth cones of BDNF-dependent neurons turn toward a BDNF source. *In vivo* evidence for chemotropic effects of neurotrophins is primarily from studies in which NGF application or transgene-driven expression evoked massive in-growth of sympathetic fibers to inappropriate tissues (Kawaja and Crutcher, 1997; Menesini Chen *et al.*, 1978). Collectively, these studies allow for the possibility that BDNF and/or NT4 are chemotropic factors for chorda tympani axons and direct taste bud target selection during development. Alternatively, BDNF and NT4 overexpression may indirectly interfere with targeting by disrupting axonal responses to other guidance cues. Cell culture studies have demonstrated that bathing neurons in BDNF can alter growth cone responses to the

guidance factors collapsin-1 (a chick semaphorin III/D homolog) (Tuttle and O'Leary, 1998) and netrin-1 (Ming *et al.*, 1999). *In vitro* evidence suggests semaphorin III/D is important for guidance of trigeminal and geniculate tongue afferents (Rochlin and Farbman, 1998; Rochlin *et al.*, 2000) and netrin is expressed in tongue epithelium (Livesey and Hunt, 1997), making both candidate guidance factors for gustatory neurons.

The misdirection of sensory axons to inappropriate targets in BDNF-OE and NT4-OE mice appears specific to the fungiform taste system because no loss of taste buds in either circumvallate or nasoincisive papillae occurred. Thus, the survival role of neurotrophins can be distinguished from a chemotropic role, depending on the end organ. This functional separation is also demonstrated in cutaneous touch dome mechanoreceptor end organs. Slowly adapting nerve fibers (SAI type neurons) that innervate touch domes are dependent on NT3 and lost in $NT3^{-/-}$ mice (Airaksinen *et al.*, 1996). Conversely, mice that overexpress NT3 have more sensory neurons and larger touch domes containing increased nerve fiber density relative to control mechanoreceptor units (Albers *et al.*, 1996; Krimm *et al.*, 2000). Although more fibers were found in the skin, inappropriate target innervation was never observed, suggesting that NT3, although important for SAI neuron survival, did not influence SAI target selection. Thus, overlapping chemotropic and survival roles for neurotrophins may be unique to a few sensory systems, which include the fungiform taste system.

BDNF and NT4 Overexpression Causes Duplication of Papillae and Taste Buds

Anatomical studies have identified taste buds that appear to be dividing during development (Bradley *et al.*, 1980), suggesting new taste buds can be added by division of preexisting taste buds. In adults, the emergence and loss of taste pores and even papillae have also been observed using videomicroscopy of living rabbit and human papillae over time (Miller, 1988; I. J. Miller, R. F. Krimm, and F. E. Reedy, unpublished observation). Evidence suggests changes in taste buds and papillae are dependent on gustatory innervation (Guagliardo *et al.*, 1999; Krimm and Hill, 1998, 2000). In this study we observed that some fungiform papillae in adult BDNF-OE and NT4-OE mice had two taste buds and that papillae occurred in clumps of two or three. Also, while most fungiform papillae lacked gustatory innervation, patches of hyperinnervation were sometimes observed. Thus, the abnormal arrangement of papillae and taste buds may have resulted if hyperinnervation of specific regions of the tongue stimulated taste bud and/or papillae division. Future experiments will test this possibility.

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