Targeted Taste Cell-specific Overexpression of Brain-derived Neurotrophic Factor in Adult Taste Buds Elevates Phosphorylated TrkB Protein Levels in Taste Cells, Increases Taste Bud Size, and Promotes Gustatory Innervation*

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Background: There is a lack of information about the roles of neurotrophin BDNF in adult taste buds.

Results: We generated taste cell-specific BDNF-overexpressing mice. Increased BDNF elevates TrkB activation, increases transcription of NCAM-TrkB, leads to larger taste buds, and increases taste cell number and taste-specific innervation.

Conclusion: BDNF has local and neuronal influences.

Significance: BDNF might be involved in the supertasting phenomenon.

Brain-derived neurotrophic factor (BDNF) is the most potent neurotrophic factor in the peripheral taste system during embryonic development. It is also expressed in adult taste buds. There is a lack of understanding of the role of BDNF in the adult taste system. To address this, we generated novel transgenic mice in which transgene expression was driven by an α-gustducin promoter coupling BDNF expression to the postnatal expression of gustducin in taste cells. Immunohistochemistry revealed significantly stronger BDNF labeling in taste cells of high BDNF-expressing mouse lines compared with controls. We show that taste buds in these mice are significantly larger and have a larger number of taste cells compared with controls. To examine whether innervation was affected in Gust-BDNF mice, we used antibodies to neural cell adhesion molecule (NCAM) and ATP receptor P2X3. The total density of general innervation and specifically the gustatory innervation was markedly increased in high BDNF-expressing mice compared with controls. TrkB and NCAM gene expression in laser capture microdissected taste epithelia were significantly up-regulated in these mice. Up-regulation of TrkB transcripts in taste buds and elevated taste cell-specific TrkB phosphorylation in response to increased BDNF levels indicate that BDNF controls the expression and activation of its high affinity receptor in taste cells. This demonstrates a direct taste cell function for BDNF. BDNF also orchestrates and maintains taste bud innervation. We propose that the Gust-BDNF transgenic mouse models can be employed to further dissect the specific roles of BDNF in the adult taste system.

More than half a century ago, pioneering work by Rita Levi Montalcini and colleagues (1) led to the discovery of growth factors. Nerve growth factor (NGF), the first growth factor discovered, is the prototype member of the neurotrophin family of neurotrophic factors. The neurotrophin family has four family members in mammals: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (2). BDNF has been shown to have a wide range of functions from trophic effect on neurons in the peripheral nervous system (3) to orchestrating the innervation of sensory organs (4–6). One such sensory organ is the chemical sensors (taste buds) in the oral cavity of mammals. Taste buds contain taste cells that are specialized epithelial cells (7) with neuronal properties; namely, releasing neurotransmitters and the ability to depolarize and form synapses (8). BDNF is expressed in taste progenitor cells prior to taste bud formation and innervation (9) and in developing taste buds, suggesting a role in gustatory innervation (6). BDNF expression continues in adult taste buds (9–11). However, its physiological role in the adult taste system is not clear. Mammalian taste cells turn over continuously throughout life (10, 11). The rapid turnover of taste receptor cells necessitates continuous formation of new connections between the nerve fibers and taste receptors cells. Taste bud development, innervation, and functional connectivity to the brain are important to understand from a developmental point of view.

Taste buds contain three distinct anatomical/histological types of elongated taste cells: type I, type II, and type III (12, 13). Type I cells are the most abundant cells in taste buds and have been suggested to have glial-like properties (8). Type II cells express T1R and T2R taste receptors and their downstream signaling components such as phospholipase Cβ2, IP3R3 (14, 15), and gustducin (16, 17). Type II cells respond to bitter, sweet taste, and umami stimuli. Type III cells respond to sour taste and carbonation (18, 19) and have synaptic contacts with the gustatory nerve fibers. They express neural cell adhesion molecule (NCAM)2 (20) as well as the synaptic membrane protein 2

The abbreviations used are: NCAM, neural cell adhesion molecule; BGH-PA, bovine growth hormone polyadenylation site; CGRP, calcitonin gene-related peptide; Gust, 7.7-kb α-gustducin promoter; LCM, laser capture microdissection; NGS, normal goat serum; PROP, 6-n-propylthiouracil.
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SNAP25 (21). Although both type II and III cells express BDNF, BDNF is expressed by a larger number of type III cells (22).

It has been shown that a 7.7-kb segment of DNA upstream to the protein coding portion of the mouse α-gustducin gene functions provides a fully functional promoter: it drove strong expression of β-galactosidase in a temporospatial pattern similar to that of endogenous α-gustducin (23). α-Gustducin is not expressed embryonically and is found in early postnatal ages in mouse taste buds (23). Thus, α-gustducin expression commences after the period of embryonic neurogenesis in gustatory and cranial ganglia has ended (24). The relationship between the gustatory neuron and the taste buds it innervates is a dynamic process, and one neuron sends branches that innervate multiple taste buds (25, 26). We and others have targeted BDNF expression to different lingual structures using nestin (27) and CK14 (28) promoters. These strategies led to ectopic embryonic expression of BDNF in lingual muscles and anterior lingual epithelial cells, respectively. With the embryonic expression, it is impossible to separate trophic effect from other functions that BDNF might have. Most importantly, BDNF was not targeted for expression in taste buds in either approach. To address these shortcomings, we utilized the α-gustducin promoter to express BDNF in postnatal taste buds to study the possible roles of BDNF in adult taste buds.

Using the α-gustducin 7.7-kb promoter we have generated transgenic mice that overexpress BDNF in taste buds of adult mice. Continued overexpression of BDNF in taste buds in postnatal mice leads to elevated levels of TrkB activation in taste cells in vivo and influences taste bud morphology and innervation, suggesting a role for BDNF in maintenance of gustatory innervation.

EXPERIMENTAL PROCEDURES
Production and Genotyping of Gust-BDNF Transgenic Mice

The 7.7-kb α-gustducin promoter (Gust) acts as a taste cell-specific promoter and to drive transgene expression in developing and adult taste buds (23). Following the promoter, three Kozak nucleotide bases, ACC (29), were added before the ATG start of the coding sequence for BDNF followed by bovine growth hormone polyadenylation site (BGH-PA). BGH-PA has been shown to successfully stabilize neurotrophin transcripts under the CK14 promoter (30). It is also commonly used in commercially available mammalian expression vectors (for instance see invitrogen.com or stratagene.com). The Kozak sequence was added to the PCR primers to amplify the full-length BDNF gene, and BGH-PA fragment was obtained from a commercially available expression vector (pcDNA 3.1; Invitrogen). The mature BDNF sequence in the transgene was sequenced several times after insertion into the construct to examine the integrity of the sequence and to prevent mutations. PCR primers were also used to sequence the transgene fragments over the ligation sites, and we have verified the exact identity of these fragments to eliminate ligation of cutoff DNA fragments. The transgene can be removed with NotI from the pBSKSII backbone (Stratagene).

Mice were generated by pronuclear microinjection of the transgene construct (α-gustducin promoter-Kozak sequence-BDNF-BGH-PA) into fertilized eggs collected from female C57BL/6J mice at the University of Michigan transgenic core facility. Procedures were approved by the Institutional Animal User Committee at the University of Michigan. The microinjected fertilized eggs were reimplanted in pseudopregnant mice. Four founder mice were generated, but three lines survived (denoted as Gust-BDNF 739, 755, and 759). Transgene expression was verified by several construct-specific PCR primers spanning the 3’ end of the promoter to 5’ regions of the mature BDNF and 3’ region of BDNF and 5’ region of the BGH-PA. No PCR product could be generated on wild-type genomic DNA. All experiments were performed on 2–4-month-old mice unless otherwise mentioned.

Histology and Immunohistochemistry

Mice were euthanized using CO2 and perfused with 2% or 4% paraformaldehyde in phosphate-buffered saline (PBS) through the ascending aorta. Tongues were dissected, postfixed for 1 h, rinsed, and stored at 4 °C in 10% sucrose until use. These procedures were approved by the Institutional Animal Care and User Committee at the University of Tennessee Health Science Center.

To measure taste bud size, immunohistochemistry was performed on 14-μm sections using the Troma-1 (rat, Hybridoma bank, 1:80) antibody. Troma-1 is a monoclonal antibody against intermediate filaments, which identifies taste buds by its reaction with cytokeratin 8 found within the taste buds (31, 32). The slides were incubated with Troma-1 overnight at 4 °C, rinsed in PBS, and incubated with cyanine-2-coupled antibody (Cy-2, 1:200; Jackson ImmunoResearch Laboratories) or with Alexa Fluor-conjugated anti-rat IgG (1:400; Molecular Probes) for 60 min at room temperature. The slides were rinsed in PBS and cover-slipped by using glycerol/PBS (1:2) mounting medium. Images were collected with a Nikon microscope (Nikon 80i, Tokyo, Japan). Taste buds were measured using ImageJ software.

BDNF and TrkB Immunohistochemistry—Gust-BDNF 739 and 759 and wild-type circumvallate papillae and brain tissues containing the hippocampal formation and cortex were sectioned at 14 μm and incubated in blocking solution with 5% normal goat serum (NGS), 1% BSA, and 1% H2O2 in 0.3% Triton-X for 40 min at room temperature. The slides were incubated for 36 h with antibodies against BDNF (1:50–1:500, rabbit; Santa Cruz Biotechnology) or phosphorylated TrkB (pTrkB, 1:100–1:500, rabbit; Abcam). Sections were rinsed in PBS and incubated with anti-rabbit Alexa Fluor 488 (1:200) or Alexa Fluor 594 (1:200) or Alexa Fluor 647 (1:100) or Alexa Fluor 750 (1:200) and Alexa Fluor 488 (1:200). Sections were thereafter incubated in diaminobenzidine substrate kit for peroxidase until the desired staining was obtained and rinsed in tap water. Slides were then dehydrated and mounted with Permount. To analyze intensity, we measured optical density of BDNF and pTrkB labeling in grayscale photomicrographs using ImageJ (National Institutes of Health) software. Black and white were set at 0 and 100%. An equal area was outlined in ImageJ with the strongest labeling in Gust-BDNF
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739 and 759 and wild-type circumvallate papillae (n = 5), and optical density was measured and expressed as percentage.

P2X3 Receptor/Troma-1—Gust-BDNF 739, 755, and 759 and wild-type circumvallate papillae were sectioned at 40 μm. The mounted tongue sections were hydrated with three 10-min washes in PBS. The slides were blocked with 5% NGS and 1% BSA for 45 min and incubated overnight with antibodies to P2X3 (1:5000, guinea pig; Chemicon) and Troma-1. These antibodies identify the purinergic P2X3 receptor and its nerve fibers and taste receptor cells, respectively. Sections were rinsed followed by incubation with anti-guinea pig Alexa Fluor 594 (1:400) and anti-rat Cy-2 (1:200) for 60–90 min.

NCAM—Gust-BDNF 739, 759, and 759 and wild-type circumvallate papillae were sectioned at 40 μm and incubated in blocking solution with 5% NGS and 1% BSA, followed by incubation with NCAM (1:400, rabbit; Chemicon). Sections were incubated with secondary antibodies for 1 h with anti-rabbit Alexa Fluor 594. NCAM is expressed on the surface of neurons and nerve fibers and type III cells in taste buds.

Confocal Microscopy—Digital images of taste buds were captured with a Zeiss laser scanning confocal microscope (LSM700) using a 20× lens. The system laser was set to excite Troma at 488 nm and NCAM at 594 nm. A pinhole size of 1.0 μm was used. The scan rate was 1 frame/s, and a total of 2 frames were averaged. Scan speed was selected at 5. Optical sections (0.7-μm spacing) were collected for the z-stack images.

Size of Fungiform and Circumvallate Taste Buds and Statistical Analysis—To examine the possible effect of BDNF overexpression on the size of fungiform and fungiform taste buds, we compared the size of taste buds in circumvallate papillae and fungiform papillae on the anterior 140 μm of the tongue in Gust-BDNF lines with wild-type mice. Tissue sections that had been incubated with Troma-1, followed by secondary antibody (Cy-2) were processed and photographed. Fungiform and circumvallate taste buds were outlined in ImageJ, and their area, maximum widths and heights for each taste bud were measured. A detailed description of the measured parameters has been reported previously (33). Statistical analysis was performed by using Student-Newman-Keuls multiple comparisons test (InStat, Graphpad) to test whether there were any significant differences in taste bud area, width/height between Gust-BDNF lines and wild-type mice. One-way analysis of variance (ANOVA) was used for statistical analysis, and a p value of ≤0.05 was considered significant.

For circumvallate taste bud measurements, the five largest taste buds from three mice were measured on a tissue section. We also counted the number of taste buds in the sections that had the highest number of taste buds. We measured the area of the inner core part of the papillae by choosing the biggest area of the section in circumvallate papillae. We also measured the width of the inner part of the circumvallate papilla and the width of the whole papillae including the trench epithelium. For fungiform taste bud measurements, the papillae at the tip of the tongue were selected (anterior 140 μm of the tongue). The 10 largest taste buds were chosen in Gust-BDNF 739, 759, and wild-type mice (n = 30).

Propidium Iodide Staining and Taste Cell Number—Following incubation with Troma-1 and Cy-2, the slides were washed three times with PBS and propidium iodide (Sigma) was then added at a final concentration of 250 μg/ml (in PBS) as described previously (34). The slides were incubated for 15 min at room temperature in dark and washed three times with PBS and coverslipped with glycerol/PBS. All taste cell nuclei were counted in five taste buds on five different slides in Gust-BDNF 739, 759, and 759 and in wild-type mice (n = 3). The calculated number of taste cells in wild type was set to 100%.

Laser Capture Microdissection (LCM), RNA Preparation, and Reverse Transcription-PCR (RT-PCR)

Adult (6 weeks and older) transgenic Gust-BDNF and wild-type mice were euthanized using CO2 asphyxiation. The circumvallate papillae were dissected, rapidly frozen on dry ice, and sectioned on a cryostat. Horizontal sections (10 μm) were collected on frosted slides and stored at −80 °C. A HistoGene LCM Frozen Section Staining kit (Arcturus) was used for dehydration and staining of the slides. HistoGene Staining Solution (Arcturus) was applied for 10 s to the tissue sections, followed by rinses in water, ethanol, and xylene. Slides were dried for 5 min, placed in a slide box containing fresh desiccant, and LCM was performed.

Arcturus Pixcell II was used for the LCM. The laser was fired on the epithelium of the circumvallate papillae containing taste buds. The tissue was collected on a cap, which was put on a 0.5-ml Eppendorf tube containing buffer (PicoPure RNA Isolation kit). Total RNA was isolated and purified from the tissue obtained by LCM using the PicoPure RNA Isolation kit (Arcturus). cDNA was synthesized (using 1 μg of RNA) by reverse transcription using the High Capacity cDNA Archive kit (Applied Biosystems) primed with random primers, allowing us to compare the levels of gene expression with the housekeeping gene cyclophilin-D. Reverse transcription was performed for 10 min at 25 °C and 120 min at 37 °C according to the manufacturer (Applied Biosystems).

Quantitative Real-time PCR

The primers and probes for the target genes were determined with the assistance of the “Universal Probe Library” (Roche Applied Science). Real-time PCR was performed using the LightCycler (Roche Applied Science) in the presence of TaqMan Probe (Universal Probe 67) according to the manufacturer’s instructions to determine BDNF, Ntrk2, and Ncam1 transcripts in the gustatory epithelium of circumvallate papillae. The primers used were bdnf forward, gcctttggagcctcctctac and reverse, ggcggccaggtaatttt; Ntrk2 forward, cgccggatgcgtttgcctcag and reverse, gcggcatccaggtaatttt; Ntrk2 forward, gcggcatccaggtaatttt; Ncam1 forward, agggaagctgtctcct and reverse, cccatctagttggtgat. Total volume reaction is 10 μl using 5 μl of TaqMan Master Mix reagent (Roche), 2 μl of cDNA (1:10), 0.2 μl of 10 μM primer pairs (forward, 5′-3′ and reverse, 3′-5′, Invitrogen), 0.1 μl of Universal probe, and 2.7 μl of RNase free water.

Thermal cycling conditions included 2 min at 50 °C, 10 min at 95 °C, and 45 cycles for 15 s at 95 °C, and 1 min at 60 °C. To normalize the amount of total BDNF, Ntrk2, and Ncam1 transcripts in each reaction, levels of cyclophilin-D were monitored in parallel samples. The assay was performed in triplicate for each sample with Gust-BDNF 739, 755, 759, and wild-type (n =
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5) and was repeated three times (biological triplicates). Results are expressed as relative levels of BDNF, Ntrk2, and Ncam1 transcripts, referred to as wild-type control samples. The amount of targets, normalized to an endogenous reference was defined by the Ct (threshold cycle) methods (Livak and Schmittgen, 2001).

Genomic DNA of Gust-BDNF and wild-type tail biopsies was purified using DNA easy-Qiagen kit. 10 μg of DNA was used for purification. 15 ng of genomic DNA from Gust-BDNF 739, 755, and 759 was used for real-time PCR to determine whether the transgenic transcript got incorporated into the genome. BDNF-primers, cyclophilin-D, and Universal probe 67 was used.

**Statistical Analysis**

The difference in the transcript expression levels of the target genes between the experimental groups were evaluated by using ANOVA.

**RESULTS**

*Generation of Transgenic Mouse Lines—*We overexpressed BDNF in postnatal taste buds to study its roles in the adult taste system, using a taste cell-specific promoter for α-gustducin. Three independent mouse lines were generated, Gust-BDNF 739, 755, and 759. The mice have been inbred for more than 10 generations, and we have achieved homozygosity in each of the transgenic mouse lines. Using mature BDNF-specific primers, we first examined the relative frequency of transgene incorporation in the genomic DNA. Normalized against wild-type mouse genomic DNA, the relative incorporation of BDNF in genomic DNA of Gust-BDNF was highest in the Gust-BDNF 755 line (Fig. 1A). To determine whether transgene copy number was a good indicator for transgene expression, we used laser capture microdissection to isolate circumvallate taste buds and gustatory epithelium from the lines, then generated cDNA. Using real-time PCR, we analyzed mature BDNF mRNA expression in circumvallate taste buds. Compared with wild-type littersmates the assays revealed 6.6-fold higher mature BDNF expression in Gust-BDNF 739, 2.0-fold higher expression in Gust-BDNF 755, and 5.7-fold higher expression in Gust-BDNF 759 (Fig. 1B). Assays were done in triplicate in three independent experiments. Our results showed that although Gust-BDNF 755 had the highest number of transgenes incorporated into its genomic DNA, the taste bud-specific mature BDNF mRNA expression was highest in Gust-BDNF 759 followed by Gust-BDNF 739. TrkB (Ntrk2) and Ncam1 transcripts were also up-regulated in the high BDNF-expressing lines (Fig. 1B, see below). These results clearly demonstrate that we have generated transgenic mice with taste bud-specific overexpression of BDNF. Due to the modest BDNF overexpression in Gust-BDNF 755 line, it was not included in subsequent analyses.

**BDNF Protein Expression and TrkB Activation Assay—**To determine whether the gustducin promoter-driven transgene affected the translational activity in taste cells and led to increased amounts of BDNF protein, we used immunohistochemistry (Fig. 2, A–F). The main selection criteria for the specific antibody was proper positive staining in cortical neurons and in the hippocampal formation and being distinct from NGF- and NT-3-like immunoreactivity in these areas (35). We screened multiple commercially available antibodies, and only one gave appropriate and reproducible results, enabling us to quantify the relative amounts of BDNF protein in wild-type and transgenic taste buds. Weak BDNF labeling was seen in taste cells of wild-type mice (Fig. 2, A and D). However, BDNF labeling was much stronger in the taste cells of Gust-BDNF mice (Fig. 2, B, C, E, and F), indicating a higher level of BDNF production in these mice than in wild-type mice. The average intensity of BDNF labeling in circumvallate taste buds in Gust-BDNF 739 (34.9%) and Gust-BDNF 759 (42.6%) was significantly higher (p < 0.0001) than in wild-type mice (19.4%) (ANOVA, post test Tukey-Kramer).

Although BDNF has been shown to orchestrate gustatory innervation, its possible roles in taste cells *per se* have not been studied. To examine BDNF function in taste cells, we studied TrkB activation and phosphorylation in taste cells. We used a well characterized antibody to phosphorylated TrkB (pTrkB) that detects both the presence and activation of this receptor (36, 37). The specificity was also verified in our experiments by evaluating pTrkB labeling in brain tissue sections. We detected phosphorylated TrkB in taste cells (Fig. 2, G and J), and brain neurons, of wild-type mice. Weak labeling was also detected in some subepithelial nerve fibers. This shows not only that taste cells express TrkB, it also indicates a local functional activation of TrkB in taste cells in response to BDNF in taste buds. Label-
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In the peripheral terminals of either gustatory or somatosensory neurons lends itself to the dual action of BDNF in taste buds and taste innervation. As mentioned previously, BDNF is overexpressed and overproduced in taste buds of Gust-BDNF mice, and thus, it would be valuable to use Gust-BDNF mice as a model to examine receptor activation and downstream signaling of BDNF in taste cells. Using the same antibody, we detected strong labeling for pTrkB in taste cells and in a larger number of taste cells in the high BDNF-expressing Gust-BDNF mice (Fig. 2, H–K). We also detected labeling in subepithelial nerve fibers. Interestingly and parallel to the increased levels of BDNF in taste buds of Gust-BDNF mice, there was a significant increase in the levels of TrkB signaling in taste cells of Gust-BDNF mice. The average intensity of pTrkB immunoreactivity in Gust-BDNF 739 (43.9%) and Gust-BDNF 759 (40.3%) was significantly higher ($p < 0.0001$) than in wild-type mice (22.2%) (ANOVA, post test Tukey-Kramer). This shows that increased levels of BDNF protein in taste buds correspond to an increase in TrkB activation and downstream signaling in taste cells.

**Increased Size of Taste Buds**—To determine whether taste buds in high BDNF-expressing transgenic mice differed in size compared with wild-type mice, we utilized the Troma-I antibody. Gustducin BDNF 739 and 759 had larger fungiform and circumvallate taste buds than wild-type control mice (Fig. 3A). The average maximum width of taste buds in circumvallate papillae of Gust-BDNF 739 (62.4 ± 2.5 μm; $p < 0.01$; taste buds = 15, $n = 3$) and Gust-BDNF 759 (67.7 ± 2.3 μm; $p < 0.001$; taste buds = 15, $n = 3$) mice are significantly wider than those in wild-type mice (52.3 ± 2.6; taste buds = 15, $n = 3$) (Fig. 3A). We also found that the average maximum surface area of fungiform taste buds (Fig. 3B) in Gust-BDNF 739 mice (28110 ± 729 μm$^2$; $p < 0.001$; taste buds = 30, $n = 3$) and Gust-BDNF 759 (29,003 ± 458 μm$^2$; $p < 0.001$; taste buds = 30, $n = 3$) are significantly larger than in wild-type mice (24,170 ± 349 μm$^2$; taste buds = 30, $n = 3$) (ANOVA, post test Tukey-Kramer).

The circumvallate papillae core appeared to have similar width in all mice. The height of taste buds (apical-basal axis

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**FIGURE 2. BDNF and pTrkB immunoreactivity in the circumvallate taste buds of wild-type and Gust-BDNF mice.** A, weak BDNF labeling is seen in the circumvallate taste buds in wild-type mice. B and C, strong BDNF labeling is observed in Gust-BDNF circumvallate papillae, indicating that BDNF levels are significantly elevated in these mice compared with wild-type controls. D–F, higher magnification of circumvallate taste buds in wild-type and Gust-BDNF mice. Arrowheads in D point to weak BDNF labeling in taste cells. A larger number of taste cells are strongly labeled in Gust-BDNF mice (E and F). G, pTrkB immunoreactivity is present in wild-type taste buds, and the labeling becomes stronger and is seen in a larger number of taste cells of Gust-BDNF transgenic mice (H and I). J–L, higher magnification of circumvallate taste buds in wild-type and Gust-BDNF mice labeled for pTrkB. A stronger pTrkB labeling is seen in a larger number of taste cells in Gust-BDNF mice (K and L) compared with wild-type controls (J). Brightness was adjusted identically in images in each row to visualize the difference in staining intensity accurately. Scale bar in A represents 200 μm in A–C and G–I. Scale bar in D represents 20 μm in D–F and J–L.
length) in the circumvallate papillae of transgenic and wild-type mice did not show a significant difference. This indicates that the gustatory epithelium thickness is similar in all mouse lines. Neither was there a statistically significant difference in the number of taste buds in circumvallate papillae (data not shown).

To examine whether the taste bud size increase in high BDNF-expressing transgenic mice was due to larger taste cells or a larger number of taste cells, we counted the number of taste cell nuclei in taste buds on tissue sections that had been stained with Troma-1 (Fig. 4, A–C). Counterstaining the cell nuclei with propidium iodide revealed 140 and 153% increased number of taste cells in Gust-BDNF 739 and 759, respectively (p < 0.001 and p < 0.001, respectively) than in wild-type mice.

We showed that the Gust-BDNF taste buds are wider in the high BDNF-expressing transgenic mice and that they contain a larger number of taste cells compared with wild-type mice (Fig. 3). Gust-BDNF mice (Fig. 5). There was much stronger labeling in Gust-BDNF mice (Fig. 5).

**P2X3 Receptor and Troma-1 Immunostaining**—We next investigated whether there was a difference in the expression of the purinergic receptor P2X3 between the transgenic and wild-type mice. P2X3 has been proposed to function as a specific marker for gustatory nerve fibers (38). Nerve fibers innervating the circumvallate taste buds stained intensely with P2X3 receptor antibodies in both wild-type and high BDNF-expressing Gust-BDNF mice (Fig. 6). There was much stronger labeling in Gust-BDNF 759 followed by Gust-BDNF 739 than in wild-type mice.

**Gene Expression in Circumvallate Taste Buds**—Because many intraepithelial NCAM-positive nerve fibers are present in the gustatory epithelia of circumvallate papillae, it makes it difficult to distinguish between immunostaining for NCAM-positive nerve fibers and NCAM-producing taste cells. In addition, to examine whether BDNF overexpression influences the morphological changes in taste buds, there is a requirement for BDNF downstream signaling. The transgene contains only the mature BDNF sequence, suggesting that only the mature protein would be overexpressed and overproduced. Thus, we also examined the taste epithelia expression of the high affinity receptor TrkB (Ntrk2) in circumvallate taste buds. Taste buds and gustatory epithelia were isolated using LCM on circumvallate papillae of 6-week-old Gust-BDNF mouse lines and their...
wild-type littermates. Real-time PCR confirmed an increase of transcript levels of BDNF, Ntrk2, and NCAM1 in Gust-BDNF 739 and 759 compared with wild-type littermates (Fig. 1B).

DISCUSSION

In the present study, we used a well characterized α-gustducin promoter (23) to drive site-specific overexpression of BDNF in taste buds. Three lines were established, two of which were high BDNF-overexpressing. We verified the increased transcriptional and translational activity of BDNF by PCR on LCM taste tissues and by immunohistochemistry on taste buds. We examined TrkB phosphorylation in response to BDNF overexpression and detected increased levels of TrkB activation in Gust-BDNF transgenic mice. High BDNF-overexpressing mice have larger taste buds and a larger number of taste cells. The larger size and cell number correspond to the increased density of the innervation in taste buds. High BDNF-overexpressing mice have hyperinnervated taste buds compared with wild-type mice. Interestingly, the increase in the gustatory innervation constitutes a major part of the general innervation density, as evaluated by immunohistochemistry to P2X3 ATP receptors. P2X3 antibodies have been suggested to identify gustatory nerve fibers in taste buds as well as some taste cells (38). In addition, there are a significantly larger number of NCAM- and pTrkB-positive cells in taste buds. NCAM is normally a marker for type III cells. This suggests that BDNF overexpression leads to an increased number of type III cells. BDNF overexpression did not cause an increased number of type II cells because phospholipase Cβ2 expression was similar to that found in wild-type mice.

NCAM-positive cells normally constitute only ~10% of the cells in mature taste buds (20). There was a clear increase in the number of NCAM-positive cells in the taste buds of the transgenic mice, as also indicated by the significant increase in the NCAM gene expression in circumvallate taste epithelia. This shows not only that NCAM protein expression is highly up-regulated, but there is also a clear taste bud-specific increase in local transcription of NCAM. Thus, we suggest that BDNF directly or indirectly increases the transcription of neuronal genes in taste buds.

A previous study showed TrkB immunoreactivity in taste buds and its co-localization with BDNF (39). In the present study, we show for the first time TrkB activation in taste cells in response to BDNF. Increased levels of BDNF in taste buds directly correlated with the elevated activation levels of TrkB. Thus, not only does BDNF positively influence transcription of
its high affinity receptor as shown in the gene expression section of this study, but also there is a higher level of TrkB phosphorylation in the taste cells of Gust-BDNF transgenic mice. We propose that Gust-BDNF mice would offer an excellent animal model to study the functional roles of BDNF and TrkB in a sensory organ that depends highly on BDNF for formation and function. In addition, there was a larger number of taste cells that contained labeling for pTrkB in Gust-BDNF taste buds, and there was an elevated level of pTrkB in the nerve fibers innervating the circumvallate papillae in these mice compared with wild-type controls. These findings suggest that BDNF is indeed involved in local taste cell-related events and in taste cell interaction with gustatory neurons. Interestingly, whereas pTrkB labeling in taste cells was altered in response to increased levels of BDNF, it was unchanged in the hippocampus and cortex, indicating taste cell specificity for α-gustducin promoter and BDNF overexpression in taste cells.

There is ample evidence that unprocessed secreted proneurotrophins function as ligands and bind to p75NTR with high affinity whereas mature neurotrophin proteins bind Trk receptors with high affinity (40). There is indeed p75NTR expression in taste buds and taste nerve fibers (41, 42). We show clearly that TrkB is active under such conditions, but its levels are elevated in Gust-BDNF mice. The simultaneous increased taste bud TrkB expression in response to increased levels of BDNF suggests that BDNF might control the expression of its own high affinity receptor in taste buds.

The relationship between BDNF and NCAM has been explored in previous studies. It was shown that NCAM phosphorylation in response to BDNF is involved in cortical neuron differentiation and survival (43). NCAM would bind BDNF to increase its concentration and participate in presentation of the ligand to its receptor TrkB. TrkB activation by BDNF would regulate many of its biological functions (44–48). Many receptor components are indeed involved in ligand concentration and presentation in the nervous system. However, it was later shown that there was a direct interaction between NCAM and TrkB molecules through their intracellular domains. TrkB acti-
viation enhanced by the presence of BDNF would lead to NCAM tyrosine phosphorylation, a process that is proposed to be involved in NCAM-mediated neurite outgrowth (49). NCAM-induced hippocampal neurite outgrowth is dependent on TrkB because reduction of TrkB expression inhibits this neurite outgrowth (50). In line with these results, our findings postulate that overexpression of BDNF might directly promote TrkB binding to NCAM through its intracellular domain and lead to tyrosine phosphorylation of NCAM and thereby modulating NCAM-mediated functions. Increased BDNF expression, as seen in high BDNF-expressing transgenic mice, promotes both TrkB and NCAM expression, suggesting that BDNF activation of TrkB promotes its expression and thus modulates the functions of a neural cell adhesion molecule possibly by direct interaction.

Another plausible mechanism for BDNF function in the peripheral taste system might be related to its function in the pain transduction and nociceptive system (51). It was shown that calcitonin gene-related peptide (CGRP) promoted P2X3 transcription in trigeminal sensory neurons. BDNF appeared also to enhance P2X3 transcription. There is a significant increase in the density of P2X3-positive nerve fibers and to some extent taste cells in high BDNF-expressing transgenic mice. Overexpressed BDNF would lead either directly to increased transcription and synthesis of P2X3 or indirectly by promoting CGRP effect in taste buds. Perigemmal sensory nerve fibers are indeed CGRP-positive (52, 53).

It has been reported that the density of fungiform papillae and fungiform taste buds correlate well with the perception of the bitter tasting compound 6-n-propylthiouracil (PROP). Perception of the bitter taste of PROP and the intensity with which human subjects rate its bitterness have led to identification of a subset of human subjects that have been called supertasters (54). Supertasters perceive an intense bitterness when exposed to PROP whereas other subjects might rate it tasteless. Not only do supertasters taste PROP, they also perceive stronger tastes from a variety of bitter and sweet compounds as well as oral irritants such as alcohol and capsaicin (54). The supertasting phenomenon in humans correlates directly with the number of taste receptors in the anterior portion of the tongue. Whereas the genetic background in rodents influences the ability of the animals to taste different compounds or require different concentrations of a compound to taste them (55, 56), there are no animal models in rodents that correspond to the phenomenon of supertasting in humans. Based on the anatomical evidence provided in the present study and our preliminary behavioral data, we propose that high BDNF-overexpressing mice may provide an animal model for supertasting. In high BDNF-overexpressing mice (i) taste buds are larger and have a larger number of taste cells and (ii) there is an increase in the density of gustatory innervation. Further studies are required to examine whether high BDNF-expressing mice might be a model for human supertasting. In addition, preliminary microarray data (data not shown) identified significant up-regulation of multiple genes involved in cell processes such as cell-cell signaling, neurogenesis, and development in high BDNF-expressing transgenic mice compared with low BDNF-expressing line and wild-type mice.

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