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Dev Biol. Author manuscript; available in PMC 2015 February 15.

Published in final edited form as:
Dev Biol. 2014 February 15; 386(2): 308–320. doi:10.1016/j.ydbio.2013.12.031.

BDNF and NT4 play interchangeable roles in gustatory development

Tao Huang[1] and **Robin F Krimm**[1]
[1]Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, KY 40292, USA

Abstract

A limited number of growth factors are capable of regulating numerous developmental processes, but how they accomplish this is unclear. The gustatory system is ideal for examining this issue because the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4) have different developmental roles although both of them activate the same receptors, TrkB and p75. Here we first investigated whether the different roles of BDNF and NT4 are due to their differences in temporal and spatial expression patterns. Then, we asked whether or not these two neurotrophins exert their unique roles on the gustatory system by regulating different sets of downstream genes. By using $Bdnf^{Nt4/Nt4}$ mice, in which the coding region for BDNF is replaced with NT4, we examined whether the different functions of BDNF and NT4 are interchangeable during taste development. Our results demonstrated that NT4 could mediate most of the unique roles of BDNF during taste development. Specifically, caspase-3-mediated cell death, which was increased in the geniculate ganglion in $Bdnf^{-/-}$ mice, was rescued in $Bdnf^{Nt4/Nt4}$ mice. In BDNF knockout mutant mice, tongue innervation was disrupted, and gustatory axons failed to reach their targets. However, disrupted innervation was rescued and target innervation is normal when NT4 replaced BDNF. Genome wide expression analyses revealed that BDNF and NT4 mutant mice exhibited different gene expression profiles in gustatory (geniculate) ganglion. Compared to wild type, the expression of differentiation-, apoptosis- and axon guidance-related genes was changed in BDNF mutant mice, which is consistent with their different roles during taste development. However, replacement of BDNF by NT4 rescued these gene expression changes. These findings indicate that the functions of BDNF and NT4 in taste development are interchangeable. Spatial and temporal differences in BDNF and NT4 expression can regulate differential gene expression *in vivo* and determine their specific roles during development.

Keywords

Taste; neurotrophins; brain derived neurotrophic factor; neurotrophin 4; geniculate ganglion neurons

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Correspondence should be addressed to: Robin F. Krimm Department of Anatomical Sciences and Neurobiology University of Louisville School of Medicine Louisville, KY 40292 Phone: (502) 852-3059 Fax: (502) 852-6228 rfkrim01@louisville.edu.

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Introduction

During development, taste neurons innervate specific regions of gustatory epithelium with a precise number of neurons. These connections are mediated by neurotrophins, including brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4). Although both BDNF and NT4 activate the same receptors, TrkB and p75, they play different regulatory roles in taste development. BDNF is expressed in epithelial placodes that eventually form fungiform papillae and taste buds and it functions as a chemoattractant allowing gustatory fibers to distinguish their fungiform papilla targets from non-gustatory epithelium, such as filiform papillae (Hoshino et al., 2010; Krimm et al., 2001; Lopez and Krimm, 2006a; Ma et al., 2009; Nosrat et al., 1997; Nosrat et al., 1996; Nosrat and Olson, 1995; Nosrat et al., 2012; Ringstedt et al., 1999). NT4 is not necessary for target innervation in the taste system (Ma et al., 2009), but is essential for geniculate ganglion neuronal survival during development (Liebl et al., 1997; Liu et al., 1995; Patel and Krimm, 2012). In mutant mice lacking either BDNF or NT4, approximately half of the neurons are lost from the geniculate ganglion (Liebl et al., 1997; Liu et al., 1995). In mice lacking both of these factors, almost all geniculate ganglion neurons are lost (Liu et al., 1995). Although both BDNF and NT4 regulate geniculate ganglion neuron number, they exert influence at different developmental stages via different mechanisms. Geniculate neurons become BDNF-dependent at E13.5, and BDNF regulates neuronal apoptosis by preventing caspase-3 activation (Patel and Krimm, 2010). Geniculate neurons become NT4-dependent by E11.5, but NT4 does not prevent caspase-3 activation (Patel and Krimm, 2012). It is unclear how BDNF and NT4 differentially regulate geniculate neuron development through the same receptors.

The diverse functions of BDNF and NT4 could be due to their distinct temporal and spatial expression patterns in the peripheral taste system during development. NT4 expression levels are the highest at embryonic day 12.5 (E12.5) and then decrease quickly, while BDNF steadily increases in the ganglia throughout embryonic development (Huang and Krimm, 2010). In the tongue, BDNF but not NT4 is specifically expressed in developing taste placodes, and expression remains high through E16.5 (Huang and Krimm, 2010; Nosrat et al., 1996). The distinct expression patterns of BDNF and NT4 may determine their different roles in taste development. Alternatively, neurotrophins can achieve diverse functions by activating different internal signaling pathways downstream of Trk receptor binding. It has been shown that Shc- and phospholipase Cγ1-dependent pathways downstream of TrkB, play distinct roles in neuronal survival, targeting innervation and synaptic plasticity (Gartner et al., 2006; Minichiello et al., 2002; Musumeci et al., 2009; Postigo et al., 2002; Sciarretta et al., 2010). BDNF and NT4 could exert different functions by activating distinct signaling pathways downstream of TrkB.

This issue of location *vs.* signaling was examined previously by replacing BDNF with NT4 (Fan et al., 2000). However, no unique roles for either BDNF or NT4 were examined in any system that utilizes both factors during development. For example, numbers of neurons were examined in the geniculate, nodose, and petrosal ganglion; but both BDNF and NT4 regulate neuron number in these ganglia. Because these knock-in mice over-express NT4, the increased NT4 expression could enhance neuron number via a variety of mechanisms without reversing the effects of BDNF removal on cell death. In fact, the neurons rescued in these mixed sensory ganglia by knocking-in NT4, may not even be the same subpopulation of neurons as those lost in *Bdnf*$^{-/-}$ mice. In addition, BDNF's unique functions like targeting were never examined. Therefore, it was still not clear if these two factors can function interchangeably during development *in vivo.* These limitations can be addressed by examining the taste system, in which BDNF and NT4 regulate neuronal survival using different mechanisms, and only BDNF regulates target innervation.

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Here, by using NT4 knock-in (*Bdnf*$^{Nt4/Nt4}$) mice (Fan et al., 2000), in which the coding region of BDNF is replaced with NT4, we determined that NT4 can replace the roles of BDNF in gustatory development. Specifically, caspase-3 mediated cell death, taste target innervation, gustatory nerve fiber branching and taste bud number are all rescued when BDNF is replaced by NT4. We then determined that BDNF and NT4 regulate different sets of genes and gene pathways, and that these expression changes are recovered when NT4 is replaced by BDNF. Taken together, our results indicate that the differing roles of BDNF and NT4 in the taste system are determined primarily by their differing timing and locations of expression.

Materials and Methods

Animals

Homozygous *Bdnf*$^{-/-}$, *Nt4*$^{-/-}$, were generated by crossing *Bdnf*$^{+/-}$ and *Nt4*$^{+/-}$ mice (Ernfors et al., 1994; Liu et al., 1995) obtained from Jackson Laboratories (Stock No. 002266 and Stock no. 002497, respectively, Bar Harbor, ME, USA). *Bdnf*$^{Nt4/Nt4}$ mice, in which the BDNF coding sequence is replaced by NT4 coding sequence and NT4 protein is produced instead of BDNF protein, were kindly provided by Dr. Fan (Fan et al., 2000). Embryos were time bred such that the plug day was designated E0.5, the following morning, males were removed from the cages, and females were examined for plugs. Ages verified based on the morphological features (Kaufman, 1995). Animals were cared for and used in accordance with the guidelines of the Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Stereology

Total numbers of geniculate ganglion neurons from *Bdnf*$^{Nt4/Nt4}$ and wild-type mice at E14.5, E16.5, and E18.5 (n = 3 per group) were estimated by the optical fractionator method using Stereo Investigator software (MBF Bioscience). Embryo heads were frozen, cut in transverse serial sections at 50-μm thickness, air dried overnight, and stained with cresyl violet. Tissues were mounted using an aqueous mounting medium (Glycergel, DAKO, Carpinteria, CA) to maintain thickness. The sampling parameters for the optical fractionator were optimized. Briefly, a contour around the geniculate ganglion was traced under 20x magnification, and neurons were counted under 100x. The counting frame size was 25 μm × 25 μm so that every frame has one to five countable neurons. The grid size was estimated to include approximately 15 counting frames in every section. The count fame depth (z-axis) was equal to minimal section thickness minus a total guard zone of 6 μm (3 μm from the top and bottom). Neurons were counted only when their nuclei came into focus. After counting, the total number of neurons was estimated for the entire volume of the geniculate ganglion, and the total volume of the ganglion was estimated using the Cavalieri method (MBF Bioscience).

Immunohistochemistry

Embryonic or newborn mice were transcardially perfused with 4% paraformaldehyde. The heads or tongues were dissected and post-fixed overnight, cryoprotected in 30% sucrose, and frozen in Optimal Cutting Temperature Compound (OCT, Tissue-Tek).

For quantification of activated caspase-3 in geniculate ganglia, E13.5 embryonic heads were sectioned at a thickness of 10 μm. Antigen retrieval was performed in citrate buffer (pH 6.0) for 15 min at 95°C. After cooling, slides were washed and incubated in 1:500 mouse anti-TUJ1 (Covance, #MMS-435P) and rabbit anti-cleaved caspase-3 antibodies (Cell Signaling Technology, #9661) in 0.3% Triton X-100 and 1% bovine serum albumin in PBS overnight.

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Following washes, sections were incubated in appropriate secondary antibodies for 2 h. Then the sections were washed, dehydrated, and coverslipped. The number of cells labeled with an antibody to cleaved caspase-3 in geniculate ganglion was counted from every third section under a fluorescent microscope. The area containing the geniculate ganglion was measured using Neurolucida software (MBF Bioscience) and multiplied by the thickness of the section to derive the volume. Then, the density of activated caspase-3-labeled cells in the geniculate ganglion was calculated for each animal.

For nerve fiber labeling, sagittal sections of E14.5 tongue were cut at 20 μm. The sections were post-fixed in 4% paraformaldehyde for 1 h. After washing with PBS, the tissue sections were blocked in 3% normal goat serum, 0.5% Triton X-100, and 0.1% NaN3 in 0.1 M PB overnight, followed by incubation in 1:500 rabbit anti-P2X3 (Millipore, AB5895) and 1:500 mouse anti-neurofilament (2H3, Developmental Studies Hybridoma Bank, University of Iowa) for 48 h. After washes in PBS, the slides were incubated in secondary antibodies (Invitrogen) overnight. The slides were then washed and mounted with DPX mounting medium (Sigma-Aldrich), and confocal images were taken within 1 week.

For taste bud quantification, we followed a previously described protocol (Patel et al., 2010). Briefly, sagittal serial sections of tongue were cut at a thickness of 16 μm. Antigen retrieval was performed as previously described. After washing, sections were incubated in 1:100 rat anti-cytokeratin-8 antibody (Troma-1, Developmental Studies Hybridoma Bank, University of Iowa). After washing with PBS, secondary antibody was applied for 2 h. Then, tissue sections were blocked in 0.01 M PBS containing 5% normal goat serum and 0.25% Triton X-100, followed by incubation in 1:1000 rabbit anti-GAP43 antibody (Chemicon, #AB5220) overnight. The slides were rinsed and incubated in secondary antibody (Invitrogen) for 2 h. After washing, the slides were dehydrated, cleared in CitriSolv, and coverslipped using DPX mounting medium (Fluka). Taste buds in the anterior tongue were counted under a fluorescent microscope (Leica).

Geniculate ganglia labeling using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)

DiI-labeling was performed as described previously (Krimm et al., 2001; Lopez and Krimm, 2006a, b). Briefly, embryos were fixed in 4% paraformaldehyde, and the brain and trigeminal ganglia were removed the next day. DiI crystals (Invitrogen) were placed on the central side of the geniculate ganglion and facial nerve. Alternatively, for some embryos, DiI was placed in the middle ear, a location through which the chorda tympani projects before reaching the tongue. Embryos were placed into 4% paraformaldehyde and incubated at 37°C for 2–10 weeks depending on the age of the embryo. The DiI-labeled tongue was photographed using a Leica MZFL fluorescent dissecting microscope equipped with a QImaging Retiga EXi CCD camera. Images were collected from tongues of $Bdnf^{-/-}$ ($n = 9$), $Bdnf^{Nt4/Nt4}$ ($n = 24$), and wild-type mice ($n = 35$) at E14.5, E16.5, and E18.5. After they were photographed, the DiI-labeled tongues were processed for confocal imaging and branching quantification. The remaining portion of the head, which included the geniculate ganglion, was frozen in OCT embedding medium and stored at −80°C for stereology.

Neural buds at age E16.5 and E18.5 were quantified from photomicrographs of the dorsal tongue surface. All of the neural buds on the anterior two-thirds of the tongue were included but those on the ventral surface were not. Quantification was conducted with the experimenter blind to experimental genotype.

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Branching quantification

Taste fiber branching in the tongue from *Bdnf*$^{-/-}$ (*n* = 3), *Bdnf*$^{Nt4/Nt4}$ (*n* = 4), and wild-type mice *(n* = 7*)* was quantified at E16.5 following previously published protocol (Ma et al., 2009). Briefly, confocal images of 425-μm squares from the dorsal-half of the tongue mid-region were captured with a *Z* step of 1 μm beginning at the epithelial surface and continuing to a depth of 100 μm below the tongue surface. Neurolucida software (MBF Bioscience) was used to trace each fiber bundle beginning at its most ventral point in the prism and continuing until all the branches were traced. Data collected from these tracings included the number of branch points, the number of terminal branches, and the total length of the combined fiber bundles, all of which reflect the degree of branching.

Laser capture microdissection and RNA isolation

The geniculate ganglion was isolated with laser capture microdissection (LCM) as previously described (Huang and Krimm, 2010). Briefly, embryo heads were sectioned (10 μm) for visual identification of the geniculate ganglion, which was captured onto CapSure Macro LCM Caps (Molecular Devices, Sunnyvale, CA, USA) under a laser capture microscope (Arcturus, Applied Biosystems, Foster City, CA, USA). For each animal, all sections containing geniculate ganglia were collected, and the captured samples were stored for RNA isolation.

Total RNA was extracted from geniculate ganglion using an RNeasy micro kit according to the manufacturer's directions (Qiagen, #74004). DNase I treatment was applied to eliminate traces of DNA during the procedure. Following isolation, RNA quality was analyzed using Bioanalyzer 2100 (Agilent Technologies). RNA Integrity Number (RIN) and 28S/18S ratio were used to estimate the RNA quality. Only RNA samples with 260/280 ratio 1.80 and RIN 8.0 were used.

Microarray analysis

A total of 24 *Bdnf*$^{-/-}$, *Nt4*$^{-/-}$, and wild-type littermates were used for microarray analysis. Each sample was pooled from two animals (two ganglia/embryo), resulting in three pooled samples from a total of six animals per group. RNA preparation procedures, hybridization, and array imaging were performed in the UofL Microarray Core Facility using established protocols. RNA amplification, fragmentation, and biotin-labeling were performed using WT-Ovation Pico RNA Amplification System (NuGen, #3300 and #4200). Biotin-labeled cDNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Raw data were obtained, and quality control was assessed using GeneChip Command Console Software and the Expression console (Affymetrix). DNA-Chip Analyzer (dChip, 2010) software (Li and Hung Wong, 2001; Li and Wong, 2001; Schadt et al., 2001; Schadt et al., 2000) was used to perform normalization and model-based expression value calculations. Different microarray samples were normalized using the baseline array that has a median probe signal, and the PM-MM difference model was applied for expression value calculation. Genes were considered to be differentially regulated if they met all 3 of the following criteria: 1) the fold change between knockout and wild-type mice exceeds 1.2; 2) the threshold for the absolute difference between knockout vs. wild type means is larger than 50; 3) the unpaired *t*-test *p*-value < 0.05. The resultant gene lists were analyzed with a web-based functional pathway analysis tool, Pathway Express, which utilizes the available Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway information (Khatri et al., 2007), to identify statistically over-represented pathways. This analysis procedure considers the list of regulated genes as a whole, which can indicate how the different conditions affect signaling processes at the cellular level. The *p*-value generated by the statistical procedures takes into account the proportion of known pathway-related genes that are represented in the gene list, but does not consider whether the genes are up- or down-regulated. To determine additional apoptosis-

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and axon guidance-related genes, the gene lists were also analyzed using Ingenuity Pathway Analysis software to include a larger set of annotated genes. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44734).

Real-time reverse-transcription PCR

Total geniculate ganglion RNA was used to synthesize cDNA using random primers (Invitrogen). The cDNA was quantified by real-time RT-PCR using QuantiTect SYBR Green PCR kit (Qiagen, #204163). The real-time PCR reactions were conducted using 10 μl total volumes with 300 nM primers. Table S1 shows the primer sequences. For normalization of cDNA loading, all samples were run in parallel with the 18S ribosomal RNA housekeeping gene. Real-time PCR was performed with ABI PRISM/7900HT Sequence detection systems (Applied Biosystems). All procedures followed the MIQE protocol of triplicate biological and technical repeats (Bustin et al., 2010). The PCR conditions were an initial incubation of 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 30 s.

Data analysis

The numbers of geniculate ganglion neurons, cleaved caspase-3-positive cells, taste buds, and axonal branching characteristics were compared among wild-type, *Bdnf*$^{-/-}$, and *Bdnf*$^{Nt4/Nt4}$ mice using one-way or two-way analysis of variance (ANOVA) followed by post hoc Bonferroni *t*-tests. For real-time PCR, the comparative $2^{-\Delta\Delta CT}$ method was used to determine target gene expression levels (Livak and Schmittgen, 2001). The gene expression levels of wild-type, *Bdnf*$^{-/-}$, and *Bdnf*$^{Nt4/Nt4}$ mice were compared using one-way ANOVA followed by post hoc *t*-tests. The significance level was set at $p < 0.05$ for all statistical comparisons.

Results

Replacement of BDNF by NT4 rescues activated caspase-3-mediated neuron loss in geniculate ganglion

BDNF and NT4 are both required for geniculate neuron survival, and each regulates different cell death genes, suggesting that they could activate different signaling pathways (Berghuis et al., 2006; Bibel and Barde, 2000; Huang and Reichardt, 2003; Minichiello et al., 1998). However, BDNF and NT4 could also function differently due to spatial and temporal differences in their expression (Huang and Krimm, 2010). To distinguish between these two possibilities, we examined whether NT4 could rescue BDNF-dependent cell death when the BDNF coding region is replaced by NT4 (Fan et al., 2000). Because increased cell death in *Bdnf*$^{-/-}$ mice results in considerable neuronal loss, we began by quantifying neuron number in the geniculate (taste) ganglion at several embryonic ages. Wild type geniculate neuron number was the same across the ages examined (Fig. 1A). This finding is similar to both our earlier results and geniculate neuron counts in rats (Carr et al., 2005; Patel and Krimm, 2010, 2012), suggesting that geniculate neurons are both lost and added at the same rates across embryonic ages. *Bdnf*$^{Nt4/Nt4}$ mice had more geniculate neurons than wild-type animals at E14.5 (1408.7 ± 164.9 *vs.* 1006.0 ± 84.9, $p < 0.001$), E16.5 (1466.2 ± 76.6 *vs.* 981.8 ± 17.6, $p < 0.001$), and E18.5 (1298.8 ± 60.9 *vs.* 1020.4 ± 33.1, $p < 0.001$) (Fig. 1A). Therefore, NT4 was more effective than BDNF at rescuing neurons from cell death. The rescue of neuronal number resulted in greater geniculate ganglion volume in *Bdnf*$^{Nt4/Nt4}$ mice than in wild-type animals at E14.5, E16.5, and E18.5 (Fig. 1B).

Although neuron survival is rescued in *Bdnf*$^{Nt4/Nt4}$ mice it is not clear if the same underlying mechanism is at work. BDNF prevents neuron apoptosis in geniculate ganglion beginning at

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E13.5 by blocking caspase-3 activation (Patel and Krimm, 2010), whereas NT4 prevents neuron death at E11.5 without blocking caspase-3 activation (Patel and Krimm, 2012). The addition of NT4 in $Bdnf^{Nt4/Nt4}$ mice might reverse caspase-3 activation, but could also rescue geniculate neurons via an alternate mechanism. To determine whether the rescue of neuron loss in $Bdnf^{Nt4/Nt4}$ mice was mediated by reversing the effects of BDNF removal, we compared the number of activated caspase-3 cells among $Bdnf^{-/-}$, $Bdnf^{Nt4/Nt4}$, and wild-type mice at E13.5 (Fig. 2), when both BDNF and NT4 support neuron survival in the geniculate ganglion (Patel and Krimm, 2010, 2012). Activated caspase-3-positive cells in $Bdnf^{-/-}$ mice geniculate ganglia were significantly increased compared with wild-type mice ($p < 0.01$; Fig. 2J). This increase was reduced in $Bdnf^{Nt4/Nt4}$ mice; in fact, the number was even lower than that in wild-type animals ($p < 0.05$; Fig. 2J). These findings indicate that replacement of BDNF with NT4 rescues neuron death in the geniculate ganglion by preventing caspase-3 activation, the same mechanism employed by BDNF, but not NT4, during normal development.

Replacement of BDNF by NT4 rescues taste target innervation

Although NT4 can replace BDNF in regulating neuron survival, it may not compensate for BDNF in regulating targeting and guidance. Neuron survival is normally regulated by both neurotrophins, but only BDNF is required for correct targeting and guidance. Targeting may utilize different signaling pathways downstream of the TrkB receptor than those employed in neuron survival (Sciarretta et al., 2010). In fact there is considerable evidence that not only do NT4 and BDNF function by activating different signaling mechanisms, neuron survival and targeting are mediated by differential signaling (Gartner et al., 2006; Ming et al., 2002; Minichiello, 2009; Minichiello et al., 2002; Postigo et al., 2002; Sciarretta et al., 2010). Therefore, it is possible that NT4 replacement of BDNF could rescue cell survival but fail to rescue target innervation. To test this possibility, we labeled geniculate ganglia of wild-type and $Bdnf^{Nt4/Nt4}$ embryos with DiI, and examined tongue innervation. During development, chorda tympani fibers navigate to fungiform papillae, defasciculate and invade the epithelium to form an expanded bulb-like termination, a “neural bud” (Lopez and Krimm, 2006b). At E14.5, when initial taste targeting occurs, neural buds were clearly visible across the anterior tongue in wild-type mice (Fig. 3A). However, no neural buds were observed in the anterior tongue by E14.5 in $Bdnf^{Nt4/Nt4}$ mice (Fig. 3B). To determine whether the lack of neural bud formation in $Bdnf^{Nt4/Nt4}$ mice was due to a delay in innervation to the tongue surface, nerve fibers were labeled using TUJ1 and P2X3 antibodies, which identify general nerve fibers and gustatory nerve fibers, respectively (Ishida et al., 2009). We found that P2X3-positive fibers invaded fungiform papillae in wild-type mice at E14.5 (Figs. 3C-D). However, by this age P2X3-positive fibers had not entered the papillae in $Bdnf^{Nt4/Nt4}$ mice (Figs. 3E-F). Thus, replacement of BDNF by NT4 delayed the timing of innervation to the tongue surface.

To determine whether the delayed innervation is recovered, the DiI-labeled tongue was also examined at later ages. At E16.5, the neural buds were clearly observed through the anterior tongue in both wild-type and $Bdnf^{Nt4/Nt4}$ mice (Fig. 4A, C), but no such neural buds were found in the tongue of $Bdnf^{-/-}$ mice (Figure 4B). Side views also revealed that the chorda tympani fibers penetrated into fungiform papillae of wild-type and $Bdnf^{Nt4/Nt4}$ mice (Fig. 4D-E). Similarly, the neural buds were also clearly visible at E18.5 (Fig. 1A-D). Although the numbers of neural buds were similar between $Bdnf^{Nt4/+}$ mice (83 ± 1.00) and wild-type (76.2 ± 5.08) at E16.5, there was a slight decrease in $Bdnf^{Nt4/Nt4}$ mice (60.3 ± 3.63) compared with wild type ($p < 0.003$). We also observed the same changes at E18.5 (63.0 ± 7.05 *vs.* 86.7 ± 4.74; $p < 0.05$). Taken together, our results indicate that replacement of BDNF by NT4 can, at least partly, rescue disrupted taste target innervation *in vivo*.

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In addition to the disrupted innervation to taste papillae, *Bdnf*$^{-/-}$ mice appeared to have more chorda tympani branching in the tongue (Fig. 4B), but branching seemed to be rescued in *Bdnf*$^{Nt4/Nt4}$ mice (Fig. 4C). To determine whether replacement of BDNF by NT4 could completely reverse the changes in the branching characteristics of chorda tympani, DiI-labeled fiber bundles in the mid-region of the tongue (Figure 5A-C) were traced through a confocal image stack from ventral to dorsal (Figs. 5D-F). Branch ends and points, where single fiber bundles bifurcated or trifurcated, were counted. In *Bdnf*$^{-/-}$ mice, there were approximately twice as many branch points (87.0 ± 4.62) and ends (94.0 ± 7.02) as in wild-type animals (38.4 ± 1.73 and 45.0 ± 1.84, respectively, $p < 0.001$; Fig. 5G). The total length and volume of chorda tympani fibers in *Bdnf*$^{-/-}$ mice were also significantly increased compared to wild-type animals ($p < 0.05$; Fig. 5). In *Bdnf*$^{Nt4/Nt4}$ mice, increased branching was reversed to levels lower than that observed in wild-type mice as measured by branch points and ends, and the total length ($p < 0.05$), but not by the total volume (Fig. 5G). These observations demonstrate that NT4 can replace BDNF in controlling gustatory fiber branching.

While fungiform papillae and taste bud induction may occur independently of both innervation and neurotrophin signaling (Fritzsch et al., 1997; Ito and Nosrat, 2009; Mbiene et al., 1997; Thirumangalathu et al., 2009) most taste buds require innervation for their postnatal maintenance (Hosley and Oakley, 1987; Nagato et al., 1995; Sollars, 2005). Therefore, substantial numbers of taste buds are lost by birth in *Bdnf*$^{-/-}$ mice (Ito et al., 2010; Mistretta et al., 1999; Nosrat et al., 1997; Oakley et al., 1998; Patel et al., 2010). To determine whether rescuing neural buds in *Bdnf*$^{Nt4/Nt4}$ mice also rescued taste bud development, we quantified developing taste buds at birth using anti-cytokeratin-8 as a marker (Fig. 6A-C). In *Bdnf*$^{Nt4/Nt4}$ mice, the number of fungiform taste buds was significantly higher (94.3 ± 2.73) than that observed in the BDNF mutant mice (53.0 ± 1.73) ($p < 0.001$), but it was lower than that quantified in wild-type mice (124.8 ± 2.7; p 0.002; Fig. 6D). This finding is consistent with the rescue of neural bud numbers in *Bdnf*$^{Nt4/Nt4}$ and indicates that replacement of BDNF by NT4 incompletely rescues both neural bud formation and taste bud number.

Taken together, our results indicated that removal of BDNF disrupted taste targeting and taste fiber branching in the tongue, but replacement of BDNF by NT4 restored target innervation and neural bud formation. However, it was also the case that chorda tympani innervation to the tongue surface was slightly delayed and the number of fungiform taste buds was reduced in *Bdnf*$^{Nt4/Nt4}$ mice compared with wild-type animals.

BDNF and NT4 differentially regulate gene expression during development

Given that BDNF and NT4 have differing roles in neuron survival and axon guidance during development, we speculated that they would regulate the expression of entirely different sets of genes. To examine this possibility, gene expression profile analysis by DNA microarray was performed using RNA prepared from geniculate ganglia lacking either BDNF or NT4 and the results were compared with their wild-type littermates. To maximize expression differences between wild type and knockout mice, the ganglia were collected from animals at E14.5. At this age we might expect to find expression differences in all of the following 1) factors that regulate cell death/survival, because BDNF and NT4 are still supporting geniculate neuron survival at this age (Patel and Krimm, 2010, 2012); 2) factors that regulate axon guidance and target innervation, because chorda tympani axons are first innervating their targets at E14.5 (Lopez and Krimm, 2006b; Mbiene, 2004); 3) factors that regulate neuron differentiation/function and BDNF- or NT4-dependent cell specific markers. Because many neurons have been lost by E14.5 in both knockout genotypes, we may be able to identify factors specific to BDNF- or NT4-dependent neurons. BDNF microarray data

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were verified by real-time PCR. Among 14 genes selected, the expression of 11 was successfully verified (Table S2). Note that the change in *Rock1* expression in *Bdnf*$^{-/-}$ mice was near the cutoff values ($p < 0.05$ and 1.2-fold change), but the change was confirmed by real-time PCR (Table S2), which justifies a relatively inclusive set of criteria for differences in the microarray data.

The microarray results revealed that there were 667 genes in *Bdnf*$^{-/-}$ mice with altered gene expression compared with wild-type mice. Among them, 288 were up-regulated and 379 were down-regulated. A similar number of genes (672), showed altered expression in *Nt4*$^{-/-}$ mice; 295 were up-regulated and 377 were down-regulated. Surprisingly, there were only 13 genes commonly regulated in both *Bdnf*$^{-/-}$ and *Nt4*$^{-/-}$ mice (Fig. 7A). These findings demonstrate that BDNF and NT4 function differently during development by regulating the expression of different combinations of genes.

Because BDNF and NT4 regulate different genes, we examined whether BDNF and NT4 regulate genes involved in the same biological pathways using the web-based software program Pathway-Express. In *Bdnf*$^{-/-}$ mice, 10 pathways were significantly regulated compared with wild-type mice (Table 1), and 15 pathways were regulated in *Nt4*$^{-/-}$ mice. Not surprisingly, the most impacted biological pathway in *Bdnf*$^{-/-}$ mice was axon guidance ($p = 4.2\times10^{-6}$). Consistent with our previous findings that axon guidance and taste targeting is normal in *Nt4*$^{-/-}$ mice, the axon guidance pathway was not impacted in mice lacking NT4. Although both BDNF and NT4 still prevent cell death at E14.5 (Patel and Krimm, 2012), the biological pathway for cell death was not significantly altered in either *Bdnf*$^{-/-}$ or *Nt4*$^{-/-}$ mice compared with their wild-type littermates. This could be because some factors involved in neurotrophin-regulated cell death are not yet fully integrated in the KEGG database. (Deveraux and Reed, 1999; Mincheva et al., 2011). Therefore, we also analyzed the gene lists using Ingenuity system. This analysis suggested that some additional cell death-related genes were represented in the datasets from both mutants. In all, there were nine cell death-related genes whose expression was regulated by BDNF removal and two whose expression was regulated by NT4 removal, but no overlap (Table S3). This was consistent with our earlier findings that BDNF and NT4 use different pathways to regulate cell survival (Patel and Krimm, 2010, 2012).

Replacement of BDNF by NT4 rescues the expression of genes involved in apoptosis and axon outgrowth/guidance

We demonstrated that BDNF and NT4 function differently during development, and they each regulate a different set of genes. Replacing BDNF with NT4 rescues most of the unique functions of BDNF. Therefore, we hypothesized that the replacement should also eliminate many of the gene expression changes observed in *Bdnf*$^{-/-}$ mice. To determine if the gene expression of BDNF-regulated genes is rescued by replacing BDNF with NT4, specific genes were selected not only because their expression was changed in microarray experiments, but also because of their known functions in neuronal survival and/or axon guidance. In *Bdnf*$^{-/-}$, *Bdnf*$^{Nt4/Nt4}$ and wild-type mice, expression levels of *Shc3* (Atwal et al., 2000; Postigo et al., 2002), *Chuk* (Mincheva et al., 2011), *Pou4f1* (Eng et al., 2001; Huang et al., 2001; Xiang et al., 1996), *Xiap* (Deveraux and Reed, 1999), *Cd44* (Lakshman et al., 2005; Mielgo et al., 2006), *Hoxd1* (Guo et al., 2011), *Islr2* (Mandai et al., 2009), *Dok4* (Uchida et al., 2006), and *Cntn2* (Law et al., 2008) were examined using real-time RT-PCR. According to the microarray data, the expression of these genes was not regulated by NT4. Real-time PCR experiments showed that the expression levels of all selected genes were significantly down-regulated in *Bdnf*$^{-/-}$ mice, which corroborated the microarray results (Fig. 7B). However, all of these changes were reversed in *Bdnf*$^{Nt4/Nt4}$ mice, in which expression levels were not statistically different from those in wild-type animals (Fig. 7B).

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These findings are consistent with the notion that gene expression changes in a fairly large number of BDNF-regulated genes may mediate the effects of BDNF removal on axon guidance and caspase-3-induced cell death. Consistently, the rescued gene expression may be important for the rescue of BDNF function by NT4 in $Bdnf^{Nt4/Nt4}$ mice.

Discussion

A fundamental question of developmental neurobiology is how two neurotrophins that bind to the same receptors regulate different functions. The taste system is unique because it is regulated by both BDNF and NT4, which function via the same receptors, TrkB and p75. Although both BDNF and NT4 regulate gustatory neuron survival, BDNF functions by suppressing the activation of caspase-3, while NT4 uses a different mechanism (Patel and Krimm, 2010, 2012). Both factors support fiber branching from the base of the tongue to the epithelium, but only BDNF is required for taste targeting (Ma et al., 2009). We explored the mechanisms of these different BDNF and NT4 functions by replacing BDNF with NT4 *in vivo*. Most of the effects of BDNF removal in taste development were rescued including activated caspase-3 mediated cell death, branching from the base of the tongue, and taste targeting. Next, we explored whether BDNF and NT4 removal influences expression of the same sets of genes in developing taste neurons. Gene array analysis indicated that BDNF and NT4 regulate different sets of genes and biological pathways, suggesting that the functional differences between these two factors are due to downstream transcription. This implies that BDNF and NT4 normally engage in differential signaling *in vivo*. Lastly, we determined that gene expression changes after BDNF removal are recovered in $Bdnf^{Nt4/Nt4}$ mice. These findings demonstrate that the functions of the two factors are largely interchangeable. This was surprising, because these two neurotrophic factors can activate different signaling pathway through the same Trk receptor (Berghuis et al., 2006; Bibel and Barde, 2000; Huang and Reichardt, 2003; Minichiello, 2009; Minichiello et al., 1998; Sciarretta et al., 2010). However, for the developing taste system it is primarily location and timing that dictates the function of the two TrkB ligands.

NT4 can replace BDNF in regulating neuron survival, axon branching and targeting, and gene expression

Geniculate neuron number was rescued when NT4 replaced BDNF, which is consistent with previous findings (Fan et al., 2000). However, whether NT4 rescued neuron number with the same timing or mechanism as BDNF was not previously determined. During normal development, BDNF prevents geniculate neuron death beginning at E13.5 by blocking caspase-3 activation (Patel and Krimm, 2010), while NT4 regulates cell death beginning at E11.5 via a mechanism that is caspase-3 independent (Patel and Krimm, 2012). We determined that neuron rescue by NT4 replacement of BDNF is mediated by preventing caspase-3-induced apoptosis, demonstrating that NT4 can reverse neuron loss using the same timing and mechanism normally used by BDNF. We found that *Xiap* is down-regulated in the geniculate ganglion when BDNF but not NT4 is removed; its expression recovered to wild-type levels when BDNF was replaced by NT4. XIAP is a member of the inhibitor of apoptosis family of proteins (IAP) and prevents cell apoptosis by binding to and inhibiting caspases, including caspase-3 (Deveraux and Reed, 1999; Riedl et al., 2001). TrkB ligand signaling, specifically from the target, may mediate neuronal survival by up-regulating XIAP, which blocks caspase-3 activation.

We also found that replacement of BDNF by NT4 rescues disrupted taste target innervation and the disorganized branching previously observed in BDNF knockout mice (Ma et al., 2009). The findings were interesting because there is ample *in vivo* evidence that BDNF expressed in the lingual epithelium functions as a chemoattractant allowing gustatory fibers

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to innervate their correct targets (Lopez and Krimm, 2006a; Ma et al., 2009; Nosrat et al., 1996; Nosrat and Olson, 1995; Nosrat et al., 2012; Ringstedt et al., 1999) while NT4 does not (Ma et al., 2009). However, these findings are consistent with recent *in vitro* evidence that both BDNF and NT4 are chemoattractive (Hoshino et al., 2010; Runge et al., 2012b). These data suggest that the roles of BDNF in regulating branching and target innervation are due to temporally and spatially appropriate BDNF expression. Consistent with these anatomical findings, BDNF removal changes the expression of several genes important for nerve fiber branching and targeting downstream of nerve growth factor (NGF). These genes include ISLR2, HOXD1, and CNTN2 (Guo et al., 2011; Law et al., 2008; Mandai et al., 2009). In *Bdnf*$^{Nt4/Nt4}$ mice, however, the expression changes for all three genes were recovered. This indicates that target-derived TrkB ligands initiate a transcriptional program that regulates appropriate gustatory branching and location of the correct target. This finding is particularly interesting, given that tongue-derived NT3 (Fan et al., 2004; Nosrat et al., 1996) does not influence target innervation (Nosrat et al., 1997) and cannot replace BDNF to rescue targeting deficits in *Bdnf*$^{-/-}$ mice (Agerman et al., 2003). Consistently, BDNF and NT4 can influence the functional development of geniculate neurons *in vitro* in a manner similar to each other, but completely distinct from NT3 (Al-Hadlaq et al., 2003). Therefore, while BDNF and NT4 have unique functions in gustatory development because of their spatial expression differences, NT3 functions differently due to discrepant signaling.

NT4 over-rescues most BDNF deficits

Although most BDNF functions were rescued, *Bdnf*$^{Nt4/Nt4}$ mice did not completely mimic wild-type mice. For example, there were more geniculate ganglion neurons at prenatal ages in *Bdnf*$^{Nt4/Nt4}$ mice. *Bdnf*$^{Nt4/Nt4}$ mice are NT4 overexpressors, and overexpression of NT4 in tongue epithelium is more effective than BDNF overexpression in promoting neuronal survival (Krimm et al., 2001; Lopez and Krimm, 2006a). Similarly, axon branching was over-rescued in *Bdnf*$^{Nt4/Nt4}$ mice compared to *Bdnf*$^{-/-}$ mice and the *Bdnf*$^{Nt4/Nt4}$ mice had thicker taste fiber bundles than wild-type mice. This decrease in branching in the tongue likely led to the reduction in neural buds and taste buds. These findings are consistent with *in vivo* and *in vitro* studies in which NT4 causes more nerve fiber fasciculation and less branching than BDNF (Hoshino et al., 2010; Lopez and Krimm, 2006a; Rochlin et al., 2000; Runge et al., 2012b). *Bdnf*$^{Nt4/Nt4}$ mice showed a slight delay in innervation to the tongue compared with wild-type mice. It may be that the additional NT4 at early ages suppresses neurite outgrowth; both BDNF and NT4 are capable of suppressing neurite outgrowth at high concentrations, but NT4 suppresses neurite outgrowth at lower concentrations at which BDNF promotes axon outgrowth (Runge et al., 2012b). Likewise, the overexpression of NT4 in *Bdnf*$^{Nt4/Nt4}$ mice might suppress neurite outgrowth particularly at early ages when both endogenous and BDNF promoter-controlled expression of NT4 is occurring at high levels (Huang and Krimm, 2010). At later ages endogenous NT4 levels decrease (Huang and Krimm, 2010) allowing axons to navigate to their targets. Taken together, these data can all be explained by NT4 being a more potent stimulator of TrkB than BDNF and does not require activation of different signaling pathways.

While the subtle differences between wild type and *Bdnf*$^{Nt4/Nt4}$ mice can be explained by NT4 being a more potent stimulator of TrkB-function, there are other possibilities. For example, the slight delay in innervation *Bdnf*$^{Nt4/Nt4}$ mice compared with wild-type mice, which could be caused by delayed gustatory neuron maturation. BDNF expressed in geniculate ganglion influences the maturation of geniculate neurons (Harlow et al., 2011). NT4 may be less effective than BDNF at influencing the speed at which neurons mature, resulting in delayed innervation to the tongue. It is also possible that *Bdnf*$^{Nt4/Nt4}$ mice had reduced branching compared to wild type mice because NT4 was not able to promote the local axon signaling events required for branching as effectively as BDNF. Regardless of the

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mechanisms, differences between $Bdnf^{Nt4/Nt4}$ are relatively subtle indicating that all major roles of BDNF were either partially or completely rescued by replacing BDNF with NT4. Therefore, temporal and spatial differences in signaling determine the different functions of these two neurotrophins.

Temporal and spatial regulation of gustatory development by neurotrophins

How do temporal and spatial differences in neurotrophin expression result in divergent signaling? Early in development before target innervation, NT4 is produced at high amounts in the ganglion (Huang and Krimm, 2010) and it is also more effective at stimulating neurite outgrowth than BDNF or than NT4 at later ages (Runge et al., 2012a). Once axons reach their peripheral targets NT4 expression is reduced to extremely low levels (Huang and Krimm, 2010). Both the timing and location of BDNF and NT4 expression is consistent with the idea that NT4 primarily binds to TrkB in the cell body and along the axon, where BDNF primarily binds TrkB at the terminals. Neurotrophins exert their biological effects through receptors in all of these locations (Grimes et al., 1996; Riccio et al., 1997; Watson et al., 1999). Neurotrophins in the target signal across long distances to the cell body via signaling endosomes, but signaling at the cell body or along the projection pathway may not require endosomes (Ascano et al., 2012; Cosker et al., 2008; Grimes et al., 1996; Harrington et al., 2011; Heerssen and Segal, 2002; Howe and Mobley, 2005; Zweifel et al., 2005). This difference can result in activation of different signaling pathways (Howe et al., 2001; Shao et al., 2002; Watson et al., 2001) and distinct transcriptional programs (Pazyra-Murphy et al., 2009). For example, neurotrophin signaling at the cell body activates both Erk1/2 and Erk5, while stimulation at distal axons only activates Erk5 (Shao et al., 2002; Watson et al., 2001). For the developing taste system, we might predict that NT4 is capable of stimulating endosome production and initiating the same signaling pathways when expressed in the target in $Bdnf^{Nt4/Nt4}$ mice, but NT4 does not normally signal in this manner because of location and timing of expression.

This study adds to a growing picture of how neurotrophins regulate normal taste system development. At early ages before taste axons reach their taste epithelium, the geniculate ganglion produces much higher levels of NT4 than BDNF (Huang and Krimm, 2010). At this time, TrkB-signaling at the cell body and along the projection pathway initiates a specific transcriptional program and prevents cell death (Patel and Krimm, 2012b). At later ages (E14.5), as gustatory axons reach their targets, NT4 expression in the ganglion and along the projection pathway is substantially reduced (Huang and Krimm, 2010), and neurons come in contact with BDNF produced by developing placodes. This target-derived TrkB-ligand influences both neuron survival and taste bud innervation and these roles are dictated primarily by the timing and location of its expression. TrkB-ligand from the target during target innervation initiates a transcriptional program distinctive from that of TrkB-ligands expressed much earlier from the ganglion or along the projection pathway. This transcriptional program includes factors that regulate target innervation and activated caspase-mediated cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grants DC009418 and DC007176 (R.F.K). We thank Guoping Fan for kindly providing us with the NT4 knock-in mouse line. We also thank Yinlu Chen and Jeffrey C. Petruska for assistance with microarray data analysis.

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Highlights

- NT4 can replace BDNF in regulating gustatory axon targeting
- NT4 can replace BDNF's role in preventing caspase-3-mediated cell death of gustatory neurons
- BDNF and NT4 differentially regulate gene expression in the gustatory ganglion.
- NT4 rescues apoptosis- and axon guidance-related gene expression in BDNF mutant mice
- Functions of BDNF and NT4 in taste development are interchangeable

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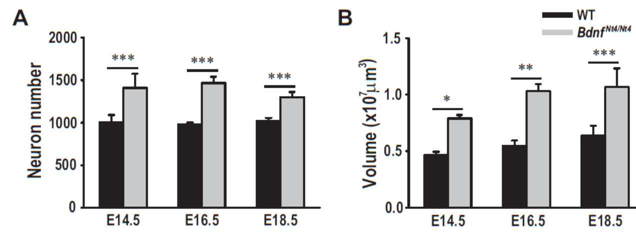

Figure 1.

Geniculate ganglion neuron number and volume were rescued to greater than wild-type values in *Bdnf*$^{Nt4/Nt4}$ mice. The number of geniculate neurons (***A***) and the volume of the geniculate ganglion (***B***) in *Bdnf*$^{Nt4/Nt4}$ mice were greater than those in wild-type mice at E14.5, E16.5, and E18.5. Values are the mean ± SEM ($n = 3$ for each group); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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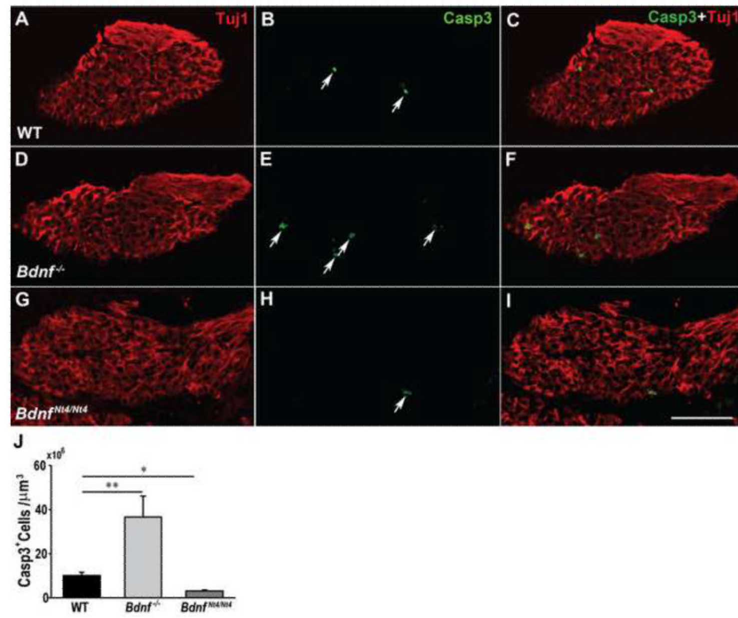

Figure 2.

BDNF removal increases activated caspase-3-positive cells (Casp3+) in geniculate ganglion at E13.5, but replacement of BDNF by NT4 reverses the increase. ***A-I***, E13.5 embryonic geniculate ganglia were double-labeled with anti-TUJ1 (red) and anti-activated caspase-3 (green). Compared with wild-type mice (***A-C***), *Bdnf*$^{-/-}$ mice (***D-F***) had more Casp3+ cells (arrows) in the geniculate ganglion. However, the change was reversed in *Bdnf*$^{Nt4/Nt4}$ mice (***G-I***). ***J***, Quantification of Casp3^{+} cells in geniculate ganglion from wild-type ($n = 6$), *Bdnf*$^{-/-}$ ($n = 3$) and *Bdnf*$^{Nt4/Nt4}$ ($n = 3$) mice; * $p < 0.05$, ** $p < 0.01$. Scale bar in ***I*** is 50 μm and applies to ***A-I***.

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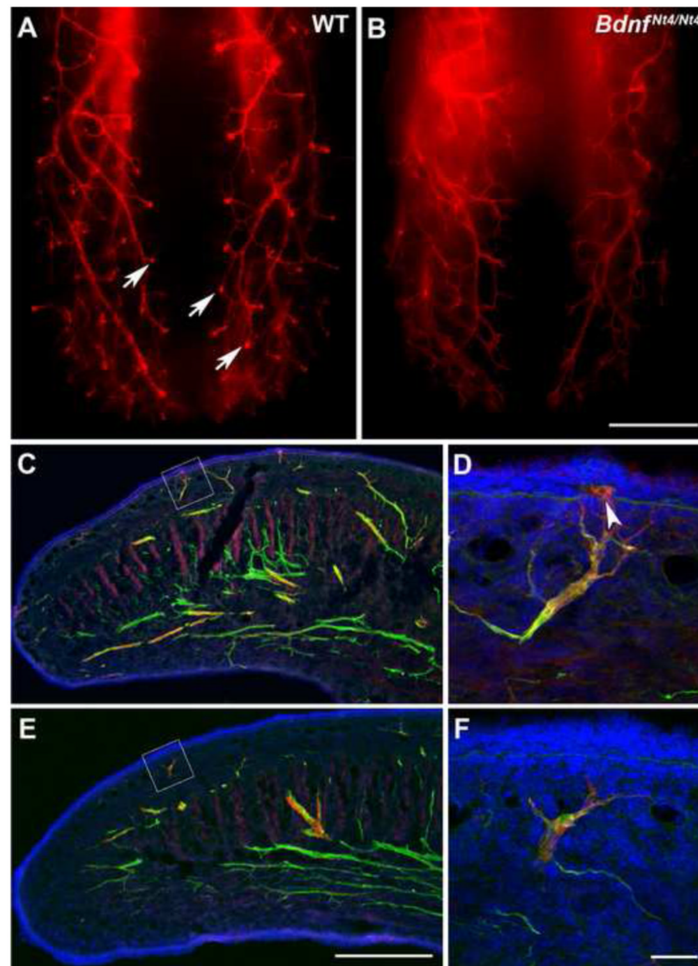

Figure 3.

Taste innervation in the anterior tongue is delayed in *Bdnf*$^{Nt4/Nt4}$ mice compared with wild type at E14.5. ***A***, Neural buds were clearly visible through the whole anterior tongue in wild-type mice (arrows). ***B***, No neural buds were observed in the anterior tongue of E14.5 *Bdnf*$^{Nt4/Nt4}$ mice. ***C-D***, Immunochemical labeling for TUJ1 (green) and P2X3 (red) and DAPI staining (blue) indicated that P2X3-positive fibers penetrated into epithelium placodes (arrow head), where taste buds will develop, in E14.5 wild-type mice. A higher magnification of the boxed image in ***C*** is shown in ***D***. ***E-F***, In *Bdnf*$^{Nt4/Nt4}$ mice, P2X3-positive fibers were below the epithelium placodes. Scale bar in ***B*** and ***E*** is 250 μm and applies to ***A-C*** and ***E***; scale bar in ***F*** is 20 μm and applies to ***D*** and ***F***.

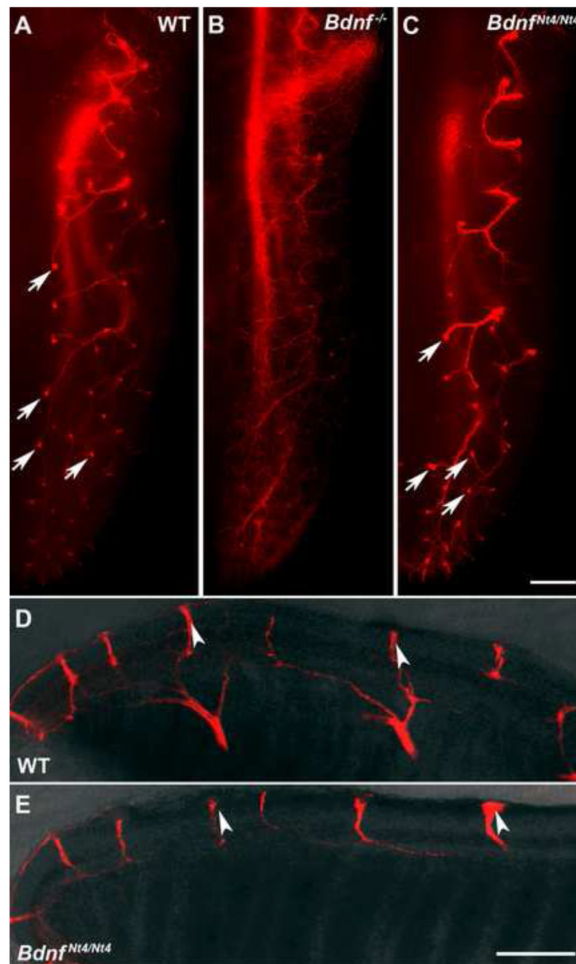

Figure 4.

Replacement of BDNF by NT4 rescues disrupted taste targeting at E16.5 and E18.5. ***A***, At E16.5, Dil-labeled chorda tympani fiber bundles ended near the tongue surface and formed neural buds (arrows) that are clearly visible from the top of the tongue in wild-type mice. ***B***, Few neural buds were observed in *Bdnf*$^{-/-}$ mice. ***C***, Similar to wild-type mice, neural buds were clearly visible (arrows) in *Bdnf*$^{Nt4/Nt4}$ mice. ***D***, ***E***, In both wild-type and *Bdnf*$^{Nt4/Nt4}$ mice, Dil-labeled chorda tympani fiber bundles penetrated the lingual epithelium at specific locations at E16.5 (arrow heads). ***F, G***, Neural buds were also clearly visible in the anterior tongues from both wild type and *Bdnf*$^{Nt4/Nt4}$ mice at E18.5. Scale bar in ***C*** is 250 μm and applies to ***A-C***; scale bar in ***E*** is 200 μm and applies to ***D*** and ***E***.

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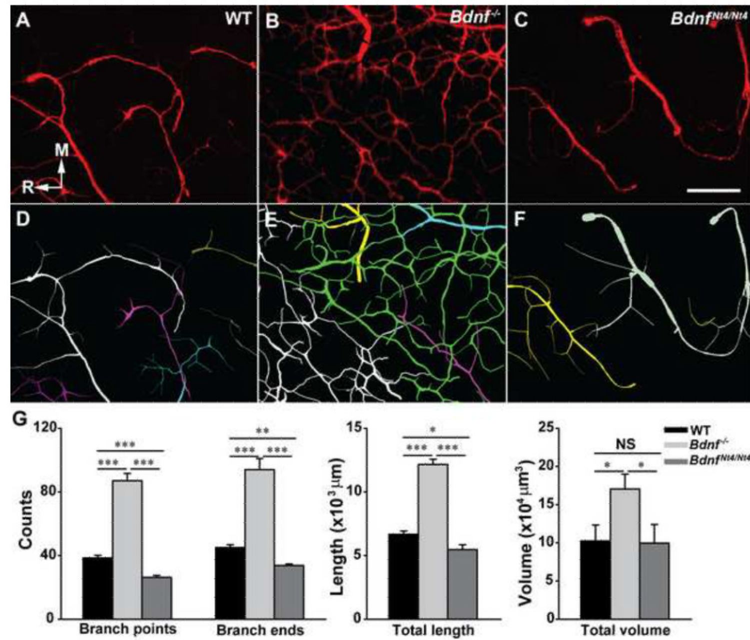

Figure 5.

Branching of chorda tympani fibers was increased in *Bdnf*$^{-/-}$ mice at E16.5 but was reduced close to wild-type levels in *Bdnf*$^{Nt4/Nt4}$ mice. ***A-C***, Confocal images of a 425 × 425-μm^2 areas from the tongue mid-region were used for branching quantification. Dil-labeled chorda tympani fiber branching was more extensive in *Bdnf*$^{-/-}$ mice (***B***) compared with that in wild-type mice (***A***), but branching in *Bdnf*$^{Nt4/Nt4}$ mice (***C***) was similar to that observed in wild-type mice. ***D-F***, For quantification, each fiber bundle in the mid-region of the tongue was traced and quantified using Neurolucida software. ***G***, Quantification of branch points, terminal branch ends, the total length and volume of all Dil-labeled fiber bundles were compared among wild-type, *Bdnf*$^{-/-}$, and *Bdnf*$^{Nt4/Nt4}$ mice ($n = 3$ embryos for each genotype). L, lateral; R, rostral. Orientation in ***A*** also applies to ***B*** and ***C***. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar in ***C*** is 100 μm and applies to ***A-F***.

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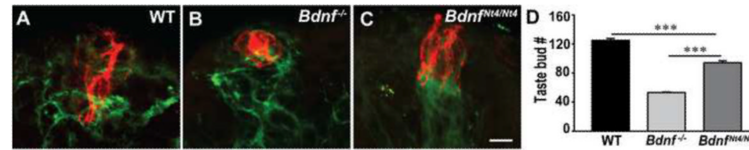

Figure 6.

Replacement of BDNF by NT4 partially rescues taste bud loss observed in $Bdnf^{-/-}$ mice. ***A-C***, Images of taste buds in the anterior tongue from newborn mice were visualized using double labeling for anti-cytokeratin-8 (red) and anti-GAP43 (green). ***D***, The numbers of taste buds in the anterior tongue were quantified for wild-type (n = 6), $Bdnf^{-/-}$ (n = 3), and $Bdnf^{Nt4/Nt4}$ (n = 3) mice. Values are the mean ± SEM. *** $p < 0.001$. Scale bar in ***C*** is 10 μm and applies to ***A-C***.

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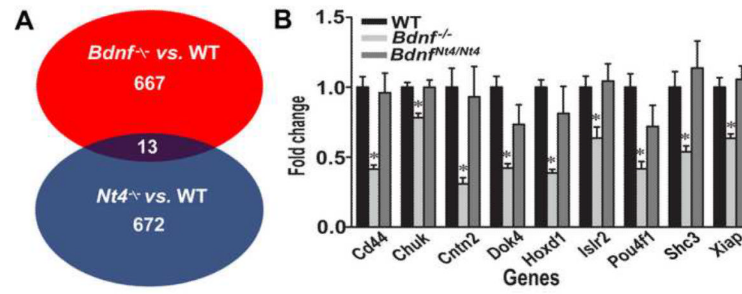

Figure 7.

BDNF and NT4 differentially regulate gene expression, but replacement of BDNF by NT4 rescues the gene expression changes. ***A***, The Venn diagram shows the numbers of genes identified in E14.5 geniculate ganglia by two microarray screens at a cutoff of $p < 0.05$ and fold change > 1.2. Only 13 genes were regulated in both *Bdnf*$^{-/-}$ and *Nt4*$^{-/-}$ mice. ***B***, Real-time PCR analysis revealed that the expression of differentiation-, apoptosis- and axon guidance-related genes was significantly down-regulated in *Bdnf*$^{-/-}$ mice (* $p < 0.05$), but expression recovered to wild-type levels in *Bdnf*$^{Nt4/Nt4}$ mice ($n = 3$).

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Table 1

Microarray analyses of E14.5 geniculate ganglion indicates that most biological pathways were differentially regulated in BDNF mutant and NT4 mutant mice.

Biological process	**%genes expected**	**BDNF KO**		**NT4 KO**	
		%genes observed	***p* value**	**%genes observed**	***p* value**
Axon guidance	2.525	**11.278**	**4.20E-06**	---	NS
Ubiquitin mediated proteolysis	2.02	**8.163**	**4.35E-04**	**6.122**	**1.59E-02**
Focal adhesion	2.357	**7.035**	**1.33E-03**	--	NS
Wnt signaling pathway	1.852	**6.962**	**3.76E-03**	--	NS
Notch signaling pathway	0.842	**8.772**	**8.60E-03**	---	NS
Glioma	1.01	**8.333**	**1.05E-02**	--	NS
ErbB signaling pathway	1.178	**7.865**	**1.17E-02**	----	NS
Adherens junction	1.01	**7.792**	**2.16E-02**	**10.39**	**1.65E-03**
Melanogenesis	1.178	**6.731**	**2.50E-02**	**8.654**	**2.66E-03**
Long-term potentiation	0.842	**6.41**	**4.72E-02**	**8.874**	**5.34E-03**
Bladder cancer	0.168	---	NS	**13.953**	**1.38E-03**
MAPK signaling pathway	1.852	---	NS	**6.273**	**1.90E-03**
Melanoma	0.673	---	NS	**9.459**	**4.95E-03**
Long-term depression	0.842	---	NS	**8.75**	**5.34E-03**
Pancreatic cancer	0.337	---	NS	**9.333**	**5.34E-03**
Tight junction	0.842	---	NS	**6.618**	**1.26E-02**
Pathways in cancer	2.357	---	NS	**5.015**	**1.58E-02**
Neuroactive ligand-receptor interaction	1.515	---	NS	**4.906**	**1.74E-02**
GnRH signaling pathway	0.842	---	NS	**7.071**	**2.03E-02**
Colorectal cancer	0.673	---	NS	**6.593**	**4.01E-02**
Cell cycle	0.842	---	NS	**5.645**	**4.67E-02**

The genes with altered expression in $Bdnf^{-/-}$ mice and $Nt4^{-/-}$ mice were analyzed by Pathway-Express software. The regulated biological processes from the Kyoto Encyclopedia of Genes and Genomes database are listed for those biological processes that were significantly ($p < 0.05$) regulated in $Bdnf^{-/-}$ mice and/or in $Nt4^{-/-}$ mice. The percentage of genes with observed changes in expression for each biological process and number of genes expected to change when a pathway was significantly impacted are listed.