Development/Plasticity/Repair

Epithelial-Derived Brain-Derived Neurotrophic Factor Is Required for Gustatory Neuron Targeting during a Critical Developmental Period

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Brain-derived neurotrophic factor (BDNF) is expressed in epithelial targets of gustatory neurons (i.e., fungiform papillae) before their innervation, and BDNF overexpression in nontaste regions of the tongue misdirects gustatory axons to these sites, suggesting that BDNF is necessary for gustatory axons to locate and innervate their correct targets during development. To test this hypothesis, we examined the targeting of taste neurons in BDNF-null mice ($bdnf^{-/-}$). Analysis of $bdnf^{-/-}$ mice using a combination of DiI labeling and electron microscopy revealed that taste regions were not innervated by gustatory axons. Instead, branching was increased and many nontaste regions were innervated. The increased branching by gustatory axons in these animals was facilitated by neurotrophin 4 (NT4), because branching was virtually eliminated in $bdnf^{-/-}/nt4^{-/-}$ mice. No abnormalities in gustatory innervation patterns and targeting were observed in $nt4^{-/-}$ mice. Conditional removal of BDNF selectively in epithelial cells disrupted targeting at the tongue tip, where gene recombination removed *bdnf* by embryonic day 13.5 (E13.5). However, innervation patterns were normal in the midregion and caudal portions of the tongue, where gene recombination did not occur until E14.5. These findings demonstrate that BDNF derived from gustatory epithelia is required for gustatory axons to correctly locate and innervate fungiform papillae. In addition, they show that BDNF-mediated targeting is restricted to a critical period of development, on or before E13.5.

Introduction

During development, taste neurons innervate gustatory regions of the tongue and palate in a highly specific manner. Gustatory regions on the developing anterior tongue can be identified by the presence of epithelial placodes, which later form fungiform papillae and taste buds (Mistretta, 1972; Farbman and Mbiene, 1991). These placodes are organized on the tongue in a precise array (Miller and Preslar, 1975), providing discrete, predictable targets for gustatory neurons. The developing gustatory placodes arise independently of innervation (Mbiene et al., 1997; Hall et al., 1999). The chorda tympani nerve innervates these placodes/papillae, whereas the greater superficial petrosal nerve innervates the gustatory regions on the soft palate. During development, chorda tympani nerves follow precise, spatially restricted pathways (Mbiene and Mistretta, 1997; Lopez and Krimm, 2006b), indicating that molecular cues produced within specific regions of the tongue control axon guidance and target innervation. Multiple attractive and repulsive cues are likely required to guide gustatory fibers from the geniculate ganglia to developing gustatory epithelia (Tessier-Lavigne and Goodman, 1996). Among these is Semaphorin 3A, which functions as a chemorepulsive molecule in the taste system (Giger et al., 1996; Rochlin and Farbman, 1998; Rochlin et al., 2000).

Brain-derived neurotrophic factor (BDNF) may be important in allowing chorda tympani fibers to identify and innervate fungiform papillae during development. BDNF is expressed in fungiform papillae before innervation, and expression occurs independently of innervation (Nosrat and Olson, 1995; Nosrat et al., 2001). The presence of BDNF at this site may enable gustatory fibers to distinguish between taste and nontaste targets during initial target innervation. BDNF is known to be an important neurotrophin for the development of the gustatory system (Conover et al., 1995; Liu et al., 1995; Nosrat et al., 1997; Mistretta et al., 1999; Sun and Oakley, 2002; Yee et al., 2003). However, the specific role of BDNF in gustatory development is not well understood.

BDNF overexpression in nongustatory tissues disrupts targeting of chorda tympani axons to fungiform papillae (Ringstedt et al., 1999; Krimm et al., 2001; Lopez and Krimm, 2006a). When BDNF is overexpressed throughout the entire lingual epithelium, chorda tympani fibers fail to innervate most fungiform papillae (Krimm et al., 2001; Lopez and Krimm, 2006a). Instead, chorda tympani fibers are attracted to filiform papillae and invade these structures (Lopez and Krimm, 2006a). It could be that BDNF produced in fungiform papillae functions as a targeting cue. However, it is possible that BDNF overexpression interferes with targeting by disrupting the response of growth cones to other guidance cues (Tuttle and O'Leary, 1998; Ming et al., 1999; Dontchev and Letourneau, 2002).

The current study was conducted to determine whether

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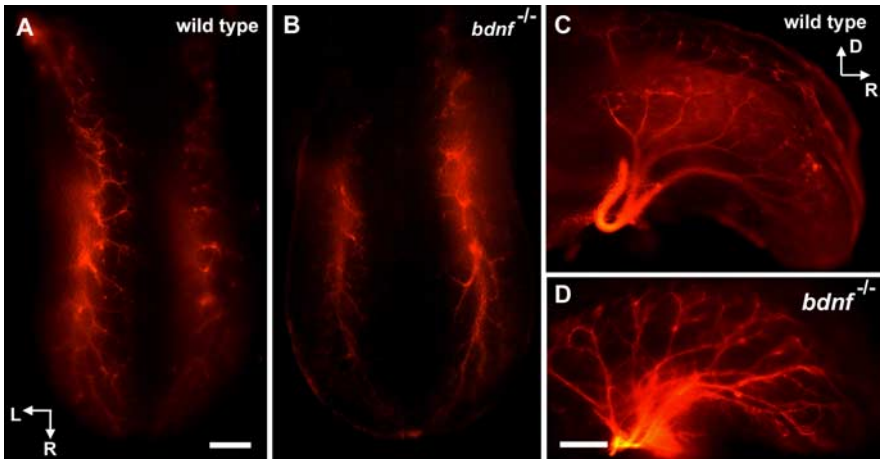

Figure 1. Innervation patterns do not differ between wild-type and $bdnf^{-/-}$ mice at E13.5. ***A–D***, In wild-type (***A***, ***C***) and $bdnf^{-/-}$ mice (***B***, ***D***), DiI-labeled chorda tympani fibers branched near the tongue surface but did not reach it (most clearly seen in ***C***). No neural buds were evident, and branching did not consistently extend the full medial-to-lateral width of the tongue surface. Individual tongues varied in the amount of branching. Orientation in ***A*** applies to ***B***. R, Rostral; L, lateral. Orientation in ***C*** also applies to ***D***. R, Rostral; D, dorsal. Scale bars: (in ***A***) ***A***, ***B***, 100 μm; (in ***D***) ***C***, ***D***, 100 μm.

BDNF is required for targeting of gustatory neurons through functional removal of this neurotrophin. We provide evidence that BDNF derived from fungiform papillae is essential for chorda tympani axon targeting and that BDNF-mediated targeting is restricted to a critical period in embryonic development.

Materials and Methods

Animals. Wild-type, $bdnf^{-/-}$, $nt4^{-/-}$, and $bdnf^{-/-}/nt4^{-/-}$ embryos were generated by crossing mice heterozygous for targeted mutations of *bdnf* and *ntf5* (*nt4*). Adult heterozygous mice were obtained from The Jackson Laboratory (stock nos. 002266 and 002497) (Ernfors et al., 1994; Liu et al., 1995). Conditional *bdnf* mutants were generated using the Cre-LoxP system. Mice containing floxed alleles ($bdnf^{lox/+}$) were obtained from The Jackson Laboratory (no. 004339) (Rios et al., 2001) and received as a kind gift from Kevin Jones (Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO) ($bdnf^{lox/+}$) (Gorski et al., 2003a,b; Baquet et al., 2005). Mice overexpressing Cre recombinase in epithelia, under the control of the Keratin-14 promoter (K14-Cre), were also obtained from The Jackson Laboratory (stock no. 004782) (Dassule et al., 2000). For some experiments, $K14\text{-}bdnf^{lox/lox}$ mice were crossed with heterozygous BDNF knock-out mice ($bdnf^{neo/+}$) to generate offspring containing all three mutated alleles ($K14\text{-}Cre;bdnf^{neo/lox}$). These mice have one functional *bdnf* allele in most locations, except where Cre is expressed in epithelial cells, including the tongue. These cells completely lack a functional *bdnf* allele after gene recombination. These mice were compared with *bdnf* heterozygous littermates that lacked Cre recombinase ($bdnf^{neo/lox}$).

For generation of embryonic mice, adult mice were bred just before the 8 h dark period. The following morning, males were removed from the cages, and females were examined for plugs. This day was designated embryonic day 0.5 (E0.5). Ages were verified based on the morphological features of each embryo stage (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.*

DiI labeling of geniculate ganglia. DiI labeling was performed as described previously (Krimm et al., 2001). Briefly, timed-bred embryos were fixed in 4% phosphate-buffered paraformaldehyde. The next day, the brain and trigeminal ganglia were removed. 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 incubated between two buffer-soaked towels for 0.5–2 h, returned to 4% paraformaldehyde, and incubated at 37°C for 1–12 weeks depending on the age of the embryo. The tongue was then dissected, examined, and photographed using a Leica MZFL fluorescent dissecting microscope equipped with either an Optonics SP Digital camera or a QImaging CE camera. Images were collected from tongues of $bdnf^{-/-}$ and wild-type mice at the following ages: E13.5 (wild type, $n = 5$; $bdnf^{-/-}$, $n = 8$), E14.5 (wild type, $n = 12$; $bdnf^{-/-}$, $n = 13$), E16.5 (wild type, $n = 9$; $bdnf^{-/-}$, $n = 9$), and E18.5 (wild type, $n = 6$; $bdnf^{-/-}$, $n = 5$). Images were collected from mice with combined *bdnf* and *nt4* null mutation at E15.5 (wild type, $n = 2$; $bdnf^{-/-}$, $n = 4$; $nt4^{-/-}$, $n = 2$; $bdnf^{-/-}/nt4^{-/-}$, $n = 4$). $Nt4^{-/-}$ were also imaged at E16.5 (wild type, $n = 3$; $nt4^{-/-}$, $n = 3$). Conditional *bdnf* mutants were imaged at E15.5 ($K14\text{-}bdnf^{neo/lox}$, $n = 7$; $bdnf^{neo/lox}$, $n = 5$; $bdnf^{+/lox}$, $n = 6$) and E16.5 ($K14\text{-}bdnf^{lox/lox}$, $n = 3$; $bdnf^{lox/lox}$, $n = 3$). Most labeled tongues were cleared in glycerol before and during imaging. After being photographed, the DiI-labeled tongues were processed for scanning electron microscopy (SEM), sectioned for confocal imaging, or split in one-half for quantification of branching.

Scanning electron microscopy. After DiI labeling and imaging, tongues were rinsed in PBS and 0.1 M cacodylate buffer. Tongues were postfixed in 1% aqueous OsO_4 for 2.0–2.5 h, washed in buffer, and successively dehydrated in a graded series of ethanol and then hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate from the tongues in a desiccator overnight. Tongues were mounted onto stubs, sputter-coated with gold, and examined by SEM (Phillips 505). Digital SEM images were captured at 130× magnification to distinguish fungiform from filiform papillae. Individual fungiform papillae were imaged at 1770× magnification.

Confocal microscopy of tongue sections. Embryonic tongues were embedded in a 10% gelatin solution and fixed overnight in 4% paraformaldehyde. Tongues were then sectioned at 50–100 μm thickness on a vibratome. Sections were mounted on slides, coverslipped in PBS, and viewed with an Olympus confocal microscope.

Quantification of branching characteristics. We quantified branching of DiI-labeled fibers at E14.5 (wild type, $n = 3$; $bdnf^{-/-}$, $n = 4$). Tongues were cut in one-half so that the dorsal one-half could be mounted on a slide. Because of the thickness of the tissue, an *in situ* hybridization frame (Eppendorf) was used to support the coverslip. Confocal images of a 500 × 450 μm rectangular area from the tongue midregion were captured with a Z step of 1 μm, beginning at the epithelial surface and continuing for a depth of 100 μm below the tongue surface (resulting in a stack of 100 images). This allowed for three-dimensional analysis of branching patterns in a 500 × 450 × 100 μm rectangular prism. These confocal stacks were analyzed with Neurolucida software (MicroBrightField) by tracing each fiber bundle beginning at its most ventral point in the cube and continuing until all the branches had been traced. The thickness of each fiber bundle and branch was recorded by adjusting the cursor width to the diameter of the fiber bundle. 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. In addition, the total volume occupied by the fibers was analyzed. This measurement, which takes into account both the length and the thickness of the combined bundles, is an indication of the total amount of innervation.

LacZ staining. Embryos were fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer for 2–4 h at 4°C. Once fixed, the tissue was rinsed several times in ice-cold PBS/$MgCl_2$ and frozen in OCT. Tissues were maintained at −80°C until staining. Whole tongues or 20 μm cryostat sections were rinsed in ice-cold PBS/$MgCl_2$ and stained at 37°C in X-gal solution containing 0.02% Igepal, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml

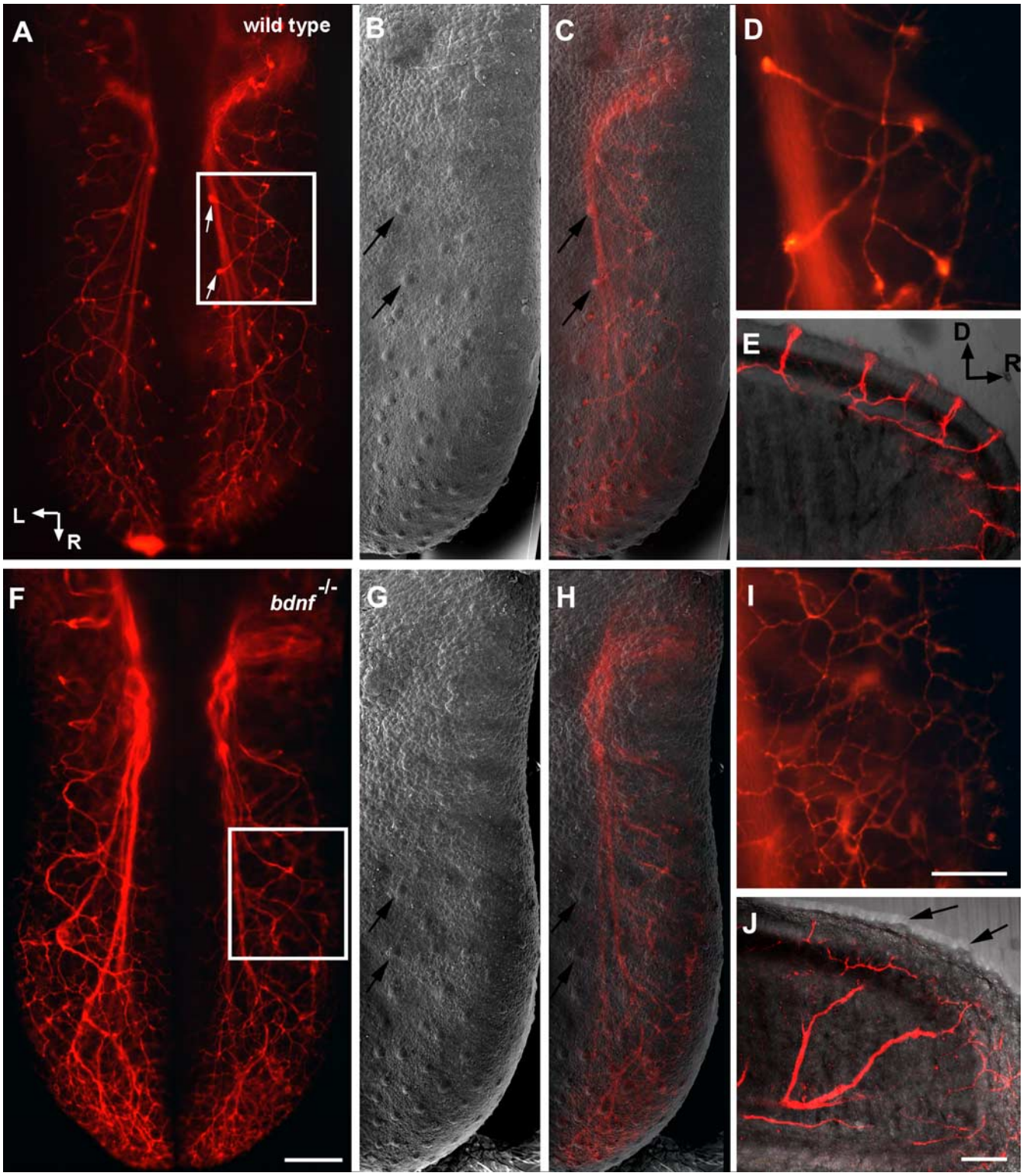

Figure 2. Chorda tympani fiber bundles fail to innervate fungiform papillae and undergo increased branching in *bdnf*$^{-/-}$ mice at E14.5. ***A–E***, In wild-type mice, individual DiI-labeled chorda tympani fiber bundles showed a stereotypical pattern of innervation. Fiber bundles ended near the tongue surface in neural buds (***A***), which colocalized with fungiform papillae (SEM micrograph of papillae shown in ***B*** and DiI labeling/SEM micrograph overlay shown in ***C***). The arrows in ***A–C*** show two examples of neural buds and corresponding fungiform papillae; a high-magnification view of these neural buds is shown in ***D***. Innervation to the epithelial surface can be seen in cross section in ***E***. ***F–J***, In *bdnf*$^{-/-}$ mice, peripheral innervation patterns were disrupted. Neural buds were not clearly observed (***F***, high-magnification view of white box in ***I***), although fungiform papillae were still present on the tongue; two examples are indicated with arrows (***G***). Papillae location did not correspond with the underlying innervation pattern (***H***). Branching was increased near the tongue surface (***I***; compared with ***D***). Chorda tympani fiber bundles did not penetrate the epithelium in specific locations (***J***, arrows indicate the locations of papillae) as they did in wild-type mice (***E***) but innervate the epithelium randomly (***J***). Orientation in ***A*** applies to ***B–D*** and ***F–I***. R, Rostral; L, lateral. Orientation in ***E*** also apples to ***J***. R, Rostral; D, dorsal. Scale bars: (in ***F***) ***A–C***, ***F–H***, 200 μm; (in ***I***) ***D***, ***I***, 100 μm; (in ***J***) ***E***, ***J***, 100 μm.

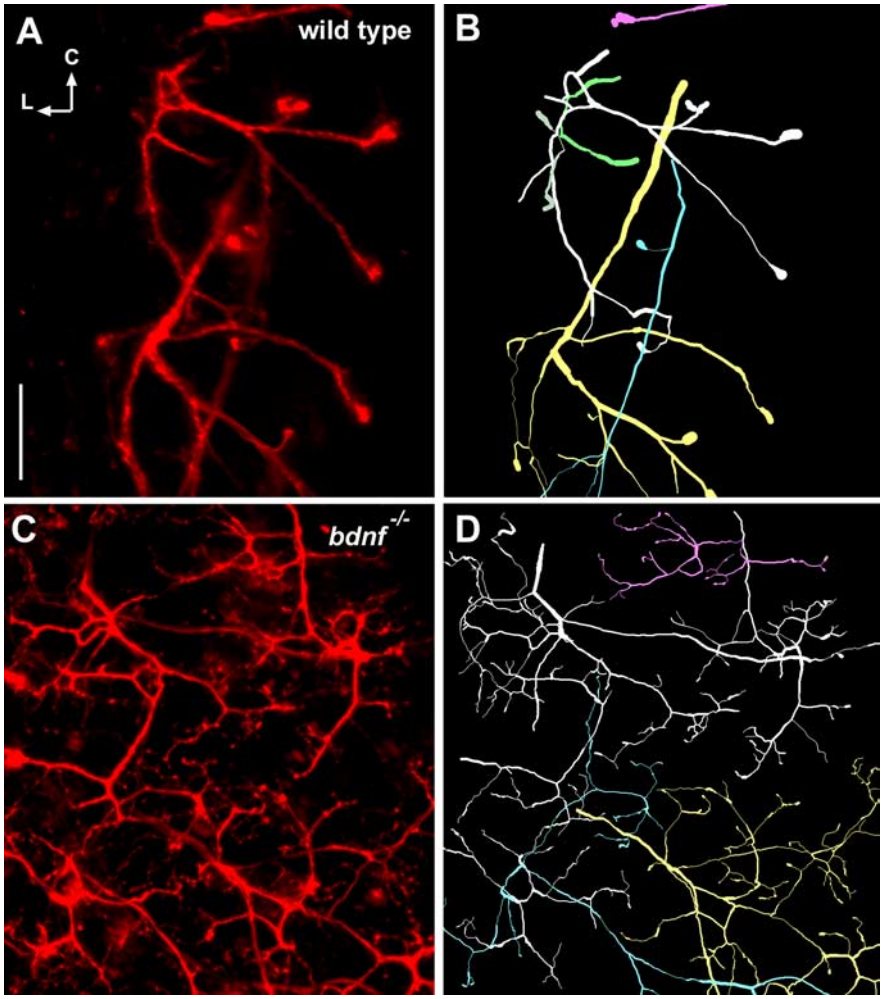

Figure 3. Chorda tympani branching is increased in *bdnf*$^{-/-}$ mice. ***A–D***, Branching of DiI-labeled fibers in the tongue midregion was more extensive in *bdnf*$^{-/-}$ mice (***C***, ***D***) than in wild-type mice (***A***, ***B***). Branching was quantified by tracing each fiber bundle (***B***, ***D***). Branches that were connected within the sampled area are displayed in the same color. Scale bar: (in ***A***) ***A–D***, 100 μm. R, Rostral; L, lateral.

X-gal in PBS. Staining was performed for 2–6 h, until color developed. Color development was observed under a dissecting microscope.

Data analysis. Landmarks on the tongue surface were used to adjust the SEM tongue image size to compensate for shrinkage of the tongue during SEM processing. The resized SEM images were overlaid onto the DiI images (Adobe Photoshop) to assess the relationship between papilla location and innervation. Papillae number in *nt4*$^{-/-}$ and wild-type mice and axonal branching characteristics in wild-type and *bdnf*$^{-/-}$ mice were compared using *t* tests. The α level was set at 0.05.

Results

BDNF-null mutation increases gustatory nerve branching and prevents the initial innervation of fungiform papillae

To determine whether BDNF is required for initial gustatory target innervation, we labeled chorda tympani axons with the lipophilic tracer, DiI, and examined innervation patterns in whole mounts of the tongue. In wild-type mice at E13.5, chorda tympani fibers had entered the tongue, branching substantially at the base of the tongue toward the epithelial surface. However, chorda tympani fibers were not associated with specific fungiform papillae and did not innervate the entire medial-to-lateral extent of the tongue surface. In addition, most chorda tympani fibers had not reached the epithelial surface. The chorda tympani innervation pattern in *bdnf*$^{-/-}$ mice did not differ from that in wild-type mice at E13.5 (Fig. 1).

Analysis of wild-type animals 1 d later (E14.5) revealed that most chorda tympani fiber bundles visible from the dorsal surface of the tongue formed an expanded bulblike termination at the tongue surface, called a "neural bud" (Fig. 2*A*,*D*) (Lopez and Krimm, 2006b). Innervation was not as clearly developed in chorda tympani fibers innervating the rostral one-fourth of the dorsal tongue and also on the ventral surface. However, the chorda tympani extended the entire medial-to-lateral extent of the epithelial surface, where fungiform papillae were located (Fig. 2*C*). On the dorsal surface of the tongue, 89% ($n = 3$) of chorda tympani nerve bundles ended at fungiform papillae (Fig. 2*A–C*). In contrast, chorda tympani fiber bundles did not specifically terminate at fungiform papillae in *bdnf*$^{-/-}$ mice. Although *bdnf*$^{-/-}$ mice had the same number of fungiform papillae on the dorsal tongue surface as wild type [86.3 $\pm$ 7.9 (SEM), $n = 3$, for *bdnf*$^{-/-}$ mice, compared with 92.0 $\pm$ 9.5, $n = 4$, for wild-type mice; $p = 0.34$], DiI and SEM overlays revealed no apparent relationship between the locations of the chorda tympani branch terminals and the fungiform papillae (Fig. 2*F–H*). Chorda tympani branching near the tongue surface appeared to increase compared with wild-type branching (Fig. 2, compare *I*, *D*). Although these fiber branches penetrated and innervated the epithelium, they innervated the lingual epithelium randomly, rather than innervating discrete locations associated with fungiform papillae (Fig. 2, compare *J*, *E*). This pattern of innervation was consistent across all 13 E14.5 *bdnf*$^{-/-}$ mice examined in this study (for other examples, see Figs. 3*C*, 4*A*). These results demonstrate that BDNF is required for chorda tympani axons to innervate fungiform papillae by E14.5 of development.

Branching in the tongue midregion of *bdnf*$^{-/-}$ mice was quantified from serial optical sections obtained with a confocal microscope (Fig. 3*A*,*C*). Branch points were identified by tracing fiber bundles through the image stack, moving from ventral to dorsal (Fig. 3*B*,*D*), and were defined as the point of division of a single fiber bundle into two fiber bundles. *Bdnf*$^{-/-}$ mice had more than twice as many branch points within the measured area than wild-type mice (76.5 $\pm$ 13.5, $n = 4$, in *bdnf*$^{-/-}$ mice, compared with 30.6 $\pm$ 4.4 in wild-type mice, $n = 3$; $p < 0.137$). The total length of neural fibers was also greater in *bdnf*$^{-/-}$ mice (7714.7 $\pm$ 1125.8 μm in *bdnf*$^{-/-}$ mice compared with 3795.0 $\pm$ 363.2 μm in wild-type mice; $n = 4$; $p < 0.035$). However, chorda tympani fiber bundles were thicker in wild-type mice than in *bdnf*$^{-/-}$ mice, as seen by a larger total nerve fascicle volume in these animals ($100.7 \pm 10.9 \times 10^3$ μm^3, $n = 3$, in wild-type mice, compared with $44.5 \pm 6.3 \times 10^3$ μm^3 in *bdnf*$^{-/-}$ mice; $n = 4$; $p < 0.005$). Therefore, whereas branching increased in *bdnf*$^{-/-}$ mice, the total amount of chorda tympani innervation in the

tongue decreased, which is consistent with the neuronal loss within the geniculate ganglion that occurs in *bdnf*$^{-/-}$ mice.

To determine whether the increased branching and failed targeting persist during later embryonic ages, we examined chorda tympani innervation patterns at E16.5 and E18.5. In *bdnf*$^{-/-}$ mice, chorda tympani innervation patterns at E16.5 were very similar to those at E14.5 (Fig. 4*A*,*B*). This pattern was consistent in all nine E16.5 *bdnf*$^{-/-}$ mice examined. However, by E18.5, *bdnf*$^{-/-}$ mice had lost much of the innervation to the tongue surface (Fig. 4*C*, in-focus innervation), causing fiber bundles below the epithelial surface to become more apparent (Fig. 4*C*, out-of-focus innervation). Examination of E18.5 *bdnf*$^{-/-}$ mouse tongue sections confirmed that some fungiform papillae were innervated (Fig. 4*D*). However, in other regions, the chorda tympani innervated areas that were devoid of fungiform papillae (Fig. 4*E*). From the surface, some remaining chorda tympani fiber bundles ended in a neural bud (Fig. 4*F*, arrows). Substantially fewer neural buds were present in the tongues of E18.5 *bdnf*$^{-/-}$ mice (32.7 ± 5.0; $n = 3$) than in those of their wild-type counterparts (93.7 ± 5.6; $n = 3$; $p < 0.001$). The small number of neural buds and the aberrant innervation made DiI-SEM alignments in these tongues inaccurate. However, the patterns of DiI-labeled neural buds and fungiform papillae were very similar in certain tongue locations (Fig. 4, compare *F*, *I*). These results indicate that increased chorda tympani nerve branching in *bdnf*$^{-/-}$ mice persisted until late during embryonic development (between E16.5 and E18.5). By E18.5, some fungiform papillae were innervated in *bdnf*$^{-/-}$ mice, which was 3–4 d after initial papillae innervation in wild-type mice.

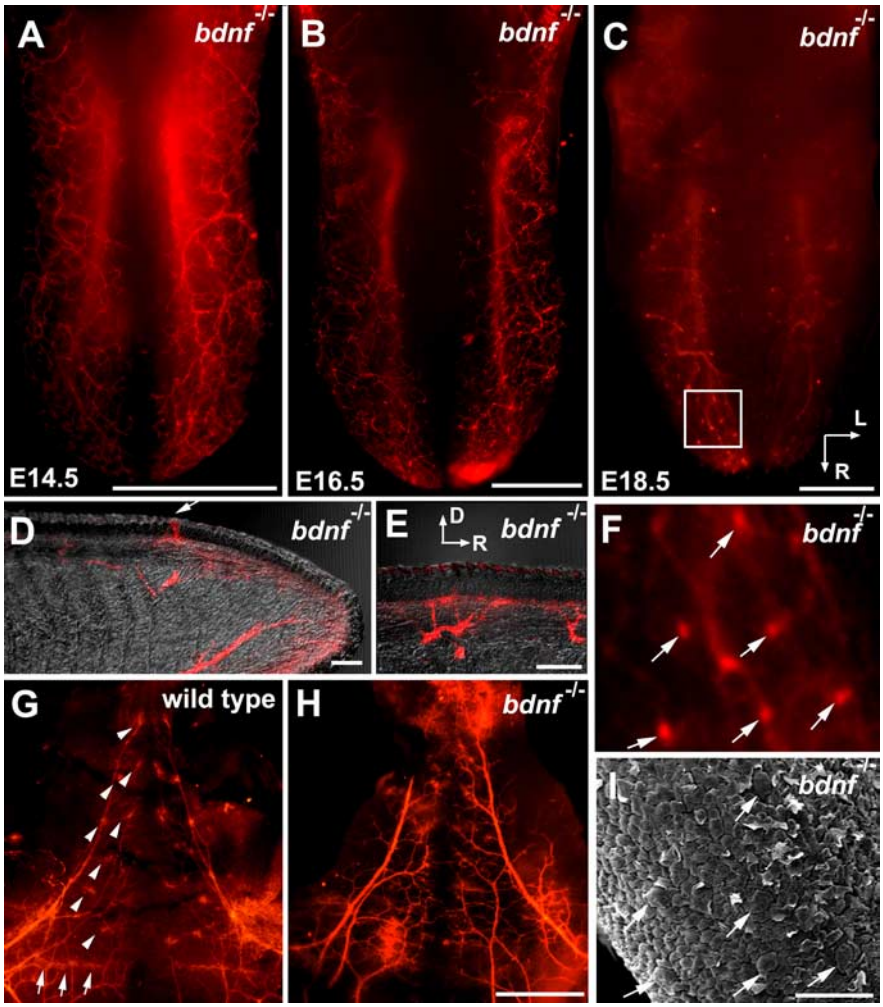

Figure 4. Innervation in *bdnf*$^{-/-}$ tongues is lost at E16.5, although some fungiform papillae remain innervated. ***A***, ***B***, DiI-labeled chorda tympani fiber bundles branched elaborately at the surface of whole *bdnf*$^{-/-}$ tongues at E14.5 (***A***) and E16.5 (***B***). ***C–F***, ***I***, By E18.5, most surface innervation was withdrawn (***C***). A higher magnification view of the E18.5 tongue tip (***F***, which corresponds to boxed area in ***C***) shows that fiber bundles ended in punctate regions. Because the depth of the tongue exceeds the focal depth of the microscope, innervation near the surface is in focus, whereas fibers deep within the tongue are not. Some fibers penetrated fungiform papillae (***D***, arrow), although aberrant innervation was present (***E***). ***I*** shows an SEM micrograph of the tongue in ***C***. Although DiI-SEM overlays in these tongues were not possible, some innervated regions (***F***, arrows) corresponded to locations of fungiform papillae (***I***, arrows). ***G***, ***H***, Specific regions of the palate corresponding to the Geschmacksstreifen (***G***, arrows). Specific regions of the posterior palatine field (***G***, arrowheads) were not innervated in *bdnf*$^{-/-}$ mice (***H***). This lack of specific innervation was apparent, although overall branching increased in the *bdnf*$^{-/-}$ palate (***H***) compared with the wild-type palate (***G***). Orientation in ***C*** applies to ***A–C*** and ***F–I***. Orientation in ***E*** also applies to ***D***. R, Rostral; D, dorsal; L, lateral. Scale bars: ***A–C***, 500 μm; ***D***, ***E***, 100 μm; (in ***I***) ***I***, ***F***, 100 μm; (in ***H***) ***G***, ***H***, 500 μm.

Neurons in the geniculate ganglion innervate taste buds on the tongue as well as taste buds on the soft palate via the greater superficial petrosal nerve (GSP). Unlike lingual taste buds, palatal taste buds do not develop in a specialized papilla. We examined GSP innervation patterns within the soft palate at E16.5 to determine whether gustatory fibers require BDNF for targeting to palatal taste buds, which are not associated with a papilla. The GSP innervates specific regions on the soft palate where taste buds will develop. Taste buds on the rostral portion of the soft palate are arranged in a row called the Geschmacksstreifen (Miller and Spangler, 1982). In the posterior palatine field, taste buds are typically arranged in scattered clumps of two or three. This very stereotypical arrangement of taste buds in the soft palate is mimicked by Sonic hedgehog labeling as early as E15.5 (Nakayama et al., 2008), before taste buds develop. We observed that, in wild-type mice, the pattern of DiI-labeled terminations in the soft palate exhibited this stereotypical arrangement by E16.5 (Fig. 4*G*, arrows/arrowheads). However, this arrangement was absent in *bdnf*$^{-/-}$ mice (Fig. 4, compare *H*, *G*). Specifically, wild-type palates contained a row of terminal endings, which corresponded to the Geschmacksstreifen (Fig. 4*G*, arrows). Also, the GSP terminated in specific widened endings in the caudal posterior palatine field (Fig. 4*G*, arrowheads), the eventual location of taste buds in the adult. In contrast, these specific terminations were absent in *bdnf*$^{-/-}$ mice (Fig. 4*H*). Thus, BDNF is required for the targeting of gustatory axons to taste bud-containing regions of the soft palate, even if no papillae are present.

NT4 supports chorda tympani branching in the tongues of *bdnf*$^{-/-}$ mice but is not required for targeting

BDNF is required for normal sensory innervation of the vestibular system and the carotid bodies (Hellard et al., 2004). However, removal of functional BDNF does not increase branching in these systems as it does in the gustatory system. Also, unlike other

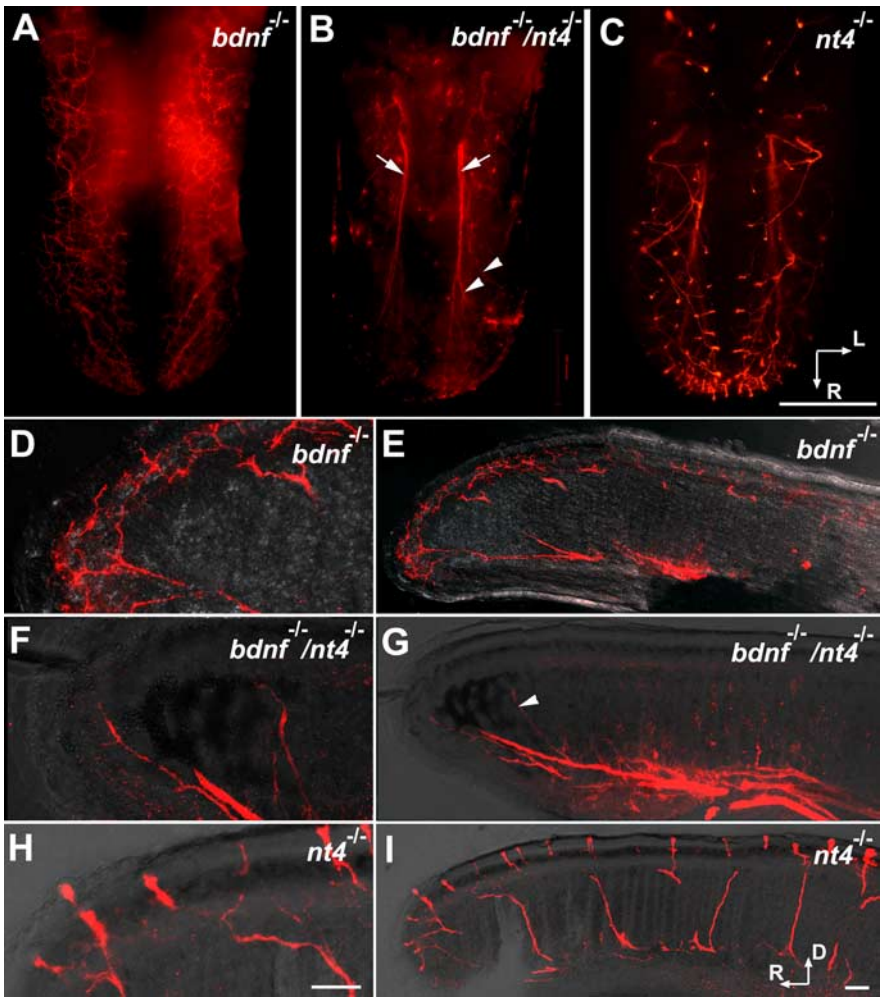

Figure 5. Most innervation of the tongue surface is lost in mice lacking both BDNF and NT4. ***A–C***, DiI-labeled whole tongues from *bdnf*$^{-/-}$ (***A***), *bdnf*$^{-/-}$/*nt4*$^{-/-}$ (***B***), and *nt4*$^{-/-}$ mice (***C***) at E15.5. ***D–I***, DiI labeling of sagittal tongue sections. In *bdnf*$^{-/-}$ mice, substantial chorda tympani branching was present near the tongue surface, but fibers did not project to the specific regions of the tongue surface to form a neural buds (***A***, ***D***, ***E***). In *bdnf*$^{-/-}$/*nt4*$^{-/-}$ mice, most chorda tympani branches at the tongue surface were eliminated (***B***, ***F***, ***G***). The absence of branching at the tongue surface allows for visualization of the chorda tympani at the tongue base, which is completely obscured in *bdnf*$^{-/-}$ mice. A few fiber bundles (***B***, arrowheads) can be seen exiting the chorda tympani at the tongue base in *bdnf*$^{-/-}$/*nt4*$^{-/-}$ mice (***B***, arrows). Innervation was normal in *nt4*$^{-/-}$ mice (***C***, ***H***, ***I***). Scale bars: (in ***C***) ***A–C***, 500 μm; (in ***H***) ***D***, ***F***, ***H***, 100 μm; (in ***I***) ***E***, ***G***, ***I***, 100 μm.

BDNF-dependent systems, gustatory neurons are also NT4 dependent (Liebl et al., 1999). Therefore, we hypothesized that the increased branching of gustatory axons in the absence of BDNF is caused by the binding of NT4 to TrkB, the common receptor for these neurotrophins. To investigate this hypothesis, we compared chorda tympani innervation patterns between E15.5 *bdnf*$^{-/-}$/*nt4*$^{-/-}$ mice and their *bdnf*$^{-/-}$ and *nt4*$^{-/-}$ counterparts. Mice were examined at E15.5 to ensure that targeting and neural bud formation had occurred in the tongue tip, the last region to be innervated. In *bdnf*$^{-/-}$ mice, the chorda tympani branched so densely at the tongue surface that the tongue base was obscured (Fig. 5*A*). Although *bdnf*$^{-/-}$/*nt4*$^{-/-}$ mice have likely lost geniculate neurons by E15.5 (Liu et al., 1995), the chorda tympani nerve was still present at the base of the tongue (Fig. 5*B*, arrows). In *bdnf*$^{-/-}$/*nt4*$^{-/-}$ tongues, only a few chorda tympani branches exited from the main chorda tympani nerve at the tongue base and extended toward the epithelial surface (Fig. 5*B*,*F*,*G*; arrowheads in *B* point to two branches that are exiting the main chorda tympani at the base). In three of the four animals examined, the few branches that were present did not reach the epithelial surface and did not branch further (Fig. 5*F*,*G*), suggesting that NT4 is required for increased branching near the epithelial surface in *bdnf*$^{-/-}$ mice. Nevertheless, one of the animals did show a small amount of innervation to the epithelium at the tongue tip. In *nt4*$^{-/-}$ mice, gustatory axon branching and target innervation were normal (Fig. 5*C*,*H*,*I*), demonstrating that NT4 is not required for targeting.

To verify that most fungiform papillae were innervated in *nt4*$^{-/-}$ mice, we compared the locations of neural buds and fungiform papillae at E16.5 (Lopez and Krimm, 2006b). We previously used this technique to assess targeting in wild-type mice at various stages of development. Unlike in *bdnf*$^{-/-}$ mice, neural buds in *nt4*$^{-/-}$ mice clearly corresponded with fungiform papillae (Fig. 6). The total number of papillae at E16.5 did not significantly differ between *nt4*$^{-/-}$ (77.7 ± 6.2) and wild-type mice (88.3 ± 4.2; $p = 0.09$). However, the number of innervated papillae was slightly smaller in *nt4*$^{-/-}$ mice (72.7 ± 6.2; $n = 3$) than in wild-type mice (86.2 ± 3.9; $n = 5$; $p < 0.04$), whereas the number of uninnervated papillae was slightly greater in *nt4*$^{-/-}$ mice (5.0 ± 0.6; $n = 3$) than in wild-type mice (2.2 ± 0.7; $n = 5$; $p < 0.02$). The number of neural buds not associated with fungiform papillae did not increase in *nt4*$^{-/-}$ mice (7.7 ± 0.3; $n = 3$) compared with wild-type mice (15.7 ± 3.4; $n = 5$; $p = 0.07$). Thus, although total lingual innervation by the chorda tympani nerve was slightly decreased in *nt4*$^{-/-}$ mice, the remaining fibers bundles were successful at innervating fungiform papillae.

Normal gustatory axon targeting requires BDNF expression in fungiform placodes by E13.5

The source of the BDNF that regulates sensory neuron targeting is not known. Removal of BDNF from the epithelial targets of gustatory neurons or nontarget derived BDNF sources (Schecterson and Bothwell, 1992; Nosrat and Olson, 1995; Nosrat et al., 1996; Nosrat et al., 1997; Yan et al., 1997) could disrupt target innervation. Thus, to determine whether epithelia-derived BDNF is specifically required for targeting, we generated epithelia-specific BDNF-null mice (Rios et al., 2001; Baquet et al., 2005). Mice possessing lox sites that flanked the BDNF coding region were mated to mice expressing Cre recombinase under the control of K14-Cre, which induces gene recombination in stratified epithelia, including oral epithelia, by E11.5 (Dassule et al., 2000). To determine when K14-Cre gene recombination would occur, we first crossed K14-Cre mice with mice carrying a lacZ reporter gene that is activated by Cre-mediated recombination (Soriano, 1999). We detected X-Gal staining in the rostral, but not the caudal, one-half of the tongue (data not shown). No X-Gal staining was observed in the soft palate. To determine the

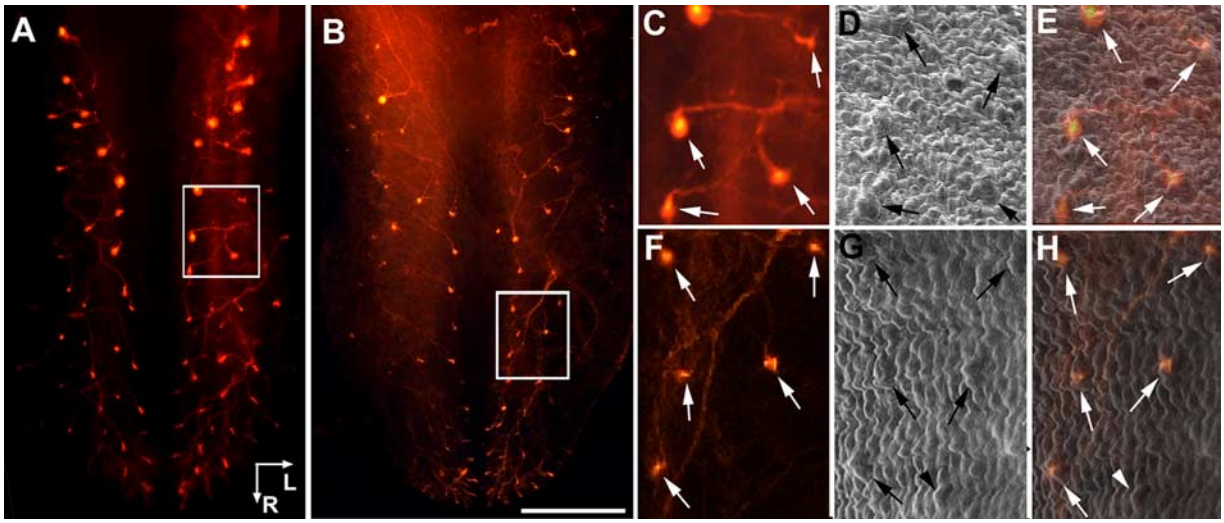

Figure 6. Removal of NT4 does not disrupt targeting. ***A***, ***B***, Chorda tympani innervation patterns were generally similar in the tongues of wild-type (***A***) and *nt4*$^{-/-}$ mice (***B***). ***C***, ***E***, Unlike in *bdnf*$^{-/-}$ tongues, clear neural buds can be seen at higher magnification in wild-type (arrows in ***C***, which corresponds to white box in ***A***) and *nt4*$^{-/-}$ tongues (arrows in ***F***, which corresponds to white box in ***B***). ***D–H***, These neural buds typically corresponded to fungiform papillae in SEM micrographs of the same wild-type (***D***, ***E***, arrows) and *nt4*$^{-/-}$ tongues (***G***, ***H***, arrows). A few fungiform papilla were not associated with a neural bud (***G***, ***H***, arrowhead). Scale bars: (in ***B***) ***A***, ***B***, 500 μm; ***C–F***, 200 μm.

precise time and location of K14-Cre-induced *bdnf* gene recombination, we crossed K14-Cre mice with mice in which lacZ is expressed after *bdnf* recombination in cells normally expressing BDNF (*K14-Cre;bdnf*$^{lacZ/+}$) (Gorski et al., 2003a,b; Baquet et al., 2005). At E13.5, a pattern of blue spots resembling fungiform placodes was present at the tongue tip, but the rest of the tongue was not well stained (Fig. 7*A*). Thus, gene recombination was not complete at the caudal end of the tongue by E13.5. At E14.5, the distribution of X-Gal staining resembled the distribution of fungiform papillae across the entire tongue surface (Fig. 7*D*). Thus, K14-Cre induces *bdnf* recombination in fungiform placodes at the tongue tip by E13.5 but does not induce gene recombination in all fungiform papillae until E14.5.

To determine whether *bdnf* recombination in the tongue tip epithelia by E13.5 or the entire tongue by E14.5 disrupted targeting of chorda tympani axons, we generated hybrid mice that contained the K14-Cre transgene, one floxed *bdnf* allele, and one *bdnf* allele with null mutation (*K14-Cre;bdnf*$^{lox/neo}$). Thus, these mice contain one functional *bdnf* allele in all cell types, except in Cre-expressing epithelial cells, which completely lacked BDNF beginning on E13.5 for the tongue tip and E14.5 for the caudal tongue. These mice were compared with heterozygous knock-outs (*bdnf*$^{+/neo}$ or *bdnf*$^{lox/neo}$) because they are likely haploinsufficient; heterozygous knock-outs have 20% fewer geniculate ganglion neurons (Fan et al., 2000), and by adulthood they are known to have slightly smaller taste buds (Yee et al., 2003). Analysis of wild-type littermates (*bdnf*$^{lox/+}$) and heterozygous BDNF knock-out littermates lacking the K14-Cre transgene (*bdnf*$^{lox/neo}$) revealed that chorda tympani target innervation was complete by E15.5. Chorda tympani innervation in the tongues of *bdnf*$^{lox/neo}$ mice was similar to that in wild-type *bdnf*$^{lox/+}$ mice, with both groups exhibiting stereotypical branching patterns (Lopez and Krimm, 2006b). Chorda tympani fiber bundles typically extended into fungiform papillae, as was most readily seen at the tip of the tongue, along the outside edge (Fig. 7*B*,*E*,*G*, arrows). In *K14-Cre;bdnf*$^{lox/neo}$ mice, innervation patterns were normal in much of the dorsal tongue surface, although BDNF was no longer expressed in these regions by E14.5 (Fig. 7*C*,*F*,*H*,*J*). A comparison of the dorsal tongue surface in *K14-Cre;bdnf*$^{lox/neo}$ and *bdnf*$^{lox/neo}$ mice revealed that neural buds were present in both (Fig. 7*B*,*C*,*H*,*J*, arrowheads). However, clear neural buds were evident on the ventral tongue tip of only *bdnf*$^{lox/neo}$ mice (Fig. 7*E*, arrowheads), indicating that innervation to the tongue tip was disrupted in *K14-Cre;bdnf*$^{lox/neo}$ mice (Fig. 7*F*). Similar to *bdnf*$^{-/-}$ mice, *K14-Cre;bdnf*$^{lox/neo}$ mice exhibited chorda tympani branching that increased at the tongue tips. Nevertheless, specific projections into fungiform papillae at the tongue tip were eliminated (Fig. 7*C*,*F*,*I*), a finding that was consistent across the seven E15.5 *K14-Cre;bdnf*$^{lox/neo}$ mice examined. To determine whether conditional removal of BDNF from the lingual epithelium when both alleles were floxed was also sufficient to disrupt chorda tympani targeting at the tongue tip, we compared E16.5 innervation patterns in *K14-Cre;bdnf*$^{lox/lox}$ and *K14-Cre;bdnf*$^{lox/lox}$ mice. Indeed, target innervation was disrupted and chorda tympani branching was increased in the tongues of *K14-Cre;bdnf*$^{lox/lox}$ mice compared with littermate controls (*bdnf*$^{lox/lox}$ mice) (Fig. 8), whose innervation patterns were normal, like wild-type mice. Like *bdnf*$^{-/-}$ mice, there was no loss of fungiform papillae on the ventral tongue surface of *K14-Cre;bdnf*$^{lox/lox}$ mice (31.7 ± 1.6) compared with littermate controls (33.5 ± 0.5; $p = 0.2$), demonstrating that the disorganized innervation pattern in the conditional *bdnf* knock-outs was not attributable to a loss of fungiform papillae. In summary, because *bdnf* gene recombination occurs in the tongue tip by E13.5, but not in the rest of the lingual epithelium until E14.5, we conclude that gene recombination at E14.5 was too late to disrupt targeting. Thus, there is a critical period on or before E13.5, by which time *bdnf* gene recombination must occur for chorda tympani targeting to be disrupted.

Discussion

During development, fungiform placodes express BDNF, whereas the surrounding epithelia does not (Nosrat and Olson, 1995; Nosrat et al., 1996). Selective BDNF expression at this site could allow gustatory axons to distinguish taste from nontaste epithelium. Here, examination of early innervation in mice lacking functional BDNF revealed that chorda tympani innervation patterns were disrupted in *bdnf*$^{-/-}$ mice, and chorda tympani

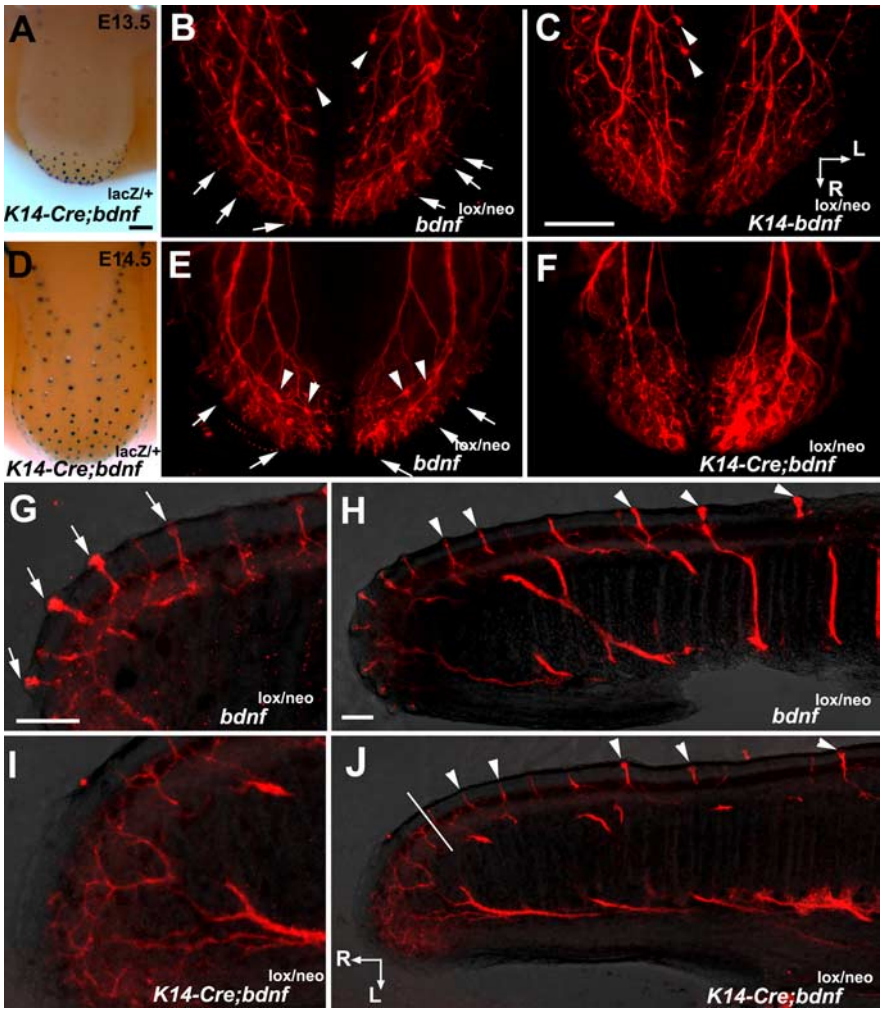

Figure 7. K14-Cre-induced *Bdnf* recombination must occur on or before E13.5 for targeting to be disrupted. ***A***, ***D***, Tongues from mice that express lacZ instead of BDNF after gene recombination. At E13.5, gene recombination had occurred at the tongue tip but was not complete in caudal portions of the tongue (***A***). At E14.5, X-Gal staining was robust in all fungiform papillae, indicating that gene recombination had occurred (***D***). ***B***, ***E***, ***G***, ***H***, DiI labeling of chorda tympani fibers in heterozygous BDNF knock-outs ($bdnf^{lox/neo}$) was normal. Neural buds were present on the dorsal surface (***B***, ***H***, arrowheads). Fibers projected to individual fungiform papillae at the tongue tip (***B***, ***E***, ***G***, arrows). In conditional BDNF knock-outs (K14-$bdnf^{lox/neo}$), innervation of the dorsal tongue surface by the chorda tympani was normal, with innervation to specific regions being apparent (***C***, ***J***, arrowheads). However, at the tongue tip, branching increased and innervation was no longer specific (***C***, ***F***, ***I***, ***J***). Scale bars: (in ***A***) ***A***, ***D***, 200 μm; (in ***C***) ***B***, ***C***, ***E***, ***F***, 250 μm; ***G*** (for ***G***, ***I***), ***H*** (for ***H***, ***J***), 100 μm.

branches failed to innervate developing fungiform papillae. Instead, chorda tympani branches innervated the tongue randomly or, alternatively, were misdirected to nontaste targets. These findings indicate that BDNF is required for gustatory fibers to locate and innervate developing fungiform papillae.

In order for BDNF to be considered a chemotropic factor that directs neurons to their targets during the final stages of target innervation, it must be both necessary and sufficient to accomplish this task. There are two lines of evidence indicating the BDNF is capable or sufficient to function as a chemotropic factor. First, BDNF overexpression in nongustatory epithelium disrupts targeting to fungiform papillae and instead directs neurons to innervate nongustatory epithelium (Lopez and Krimm, 2006a). Second, BDNF-soaked beads attract axons of the gustatory ganglion *in vitro* (Rochlin et al., 2006), showing that BDNF alone is sufficient for attracting gustatory axons toward a target. Here, we provide the first evidence that BDNF is required for targeting. Furthermore, we demonstrated that conditional removal of BDNF from the epithelium of the tongue tip by E13.5 clearly disrupts targeting in the tongue tip, demonstrating that BDNF expressed specifically within gustatory neuron targets is required for targeting of gustatory neurons to developing fungiform papillae. This finding in conditional knock-outs eliminates the possibility that failed targeting was attributable to disruption of an earlier effect of BDNF on some other aspect of neuronal development (i.e., differentiation) because geniculate neurons would still be exposed to BDNF expressed in the tongue mesenchyme, the geniculate ganglion, and the CNS (Wetmore et al., 1990; Conner et al., 1997; Furukawa et al., 1998; Nosrat, 1998; Zhou et al., 1998; Farbman et al., 2004). Together, our data plus the other studies mentioned above demonstrate that BDNF is both necessary and sufficient for attracting chorda tympani axons to fungiform papillae and encouraging innervation of these epithelial structures by gustatory fibers.

Although conditional removal of BDNF from the lingual epithelium by E13.5 disrupted targeting to the tongue tip, targeting to the rest of the tongue was unaffected, because gene recombination did not occur in these regions until E14.5. These results point to a critical period during which BDNF influences targeting. Specifically, *bdnf* recombination must occur in developing fungiform papillae by E13.5 for targeting to be disrupted. The timing of this critical period is consistent with the role of BDNF as a chemoattractant. That is, by E13.5, chorda tympani fibers are near the lingual epithelium but have not yet penetrated it, and some fiber bundles show directed growth toward fungiform papillae at this stage (Mbiene and Roberts, 2003). Thus, at E13.5, BDNF functions in the final stages of axon guidance, enabling gustatory axons to distinguish between gustatory and nongustatory epithelium as chorda tympani fibers approach the epithelial surface.

Overexpression of NT4, which binds the same receptor as BDNF, also disrupts targeting, suggesting that it too could function as a target-derived chemoattractant (Lopez and Krimm, 2006a). However, we did not observe any disruption of targeting in $nt4^{-/-}$ mice. The number of innervated fungiform papillae was slightly reduced in $nt4^{-/-}$ mice, consistent with the loss of geniculate neurons and the small loss of fungiform papillae seen by birth in these animals (Liu et al., 1995; Liebl et al., 1999). However, remaining chorda tympani fibers are associated with fungiform papillae as frequently as wild-type fibers. Furthermore, NT4-soaked beads are not chemoattractive (Rochlin et al., 2006). Therefore, NT4 does not function as a chemoattractant for gustatory axons. In the absence of BDNF, NT4 was required to support branching of the chorda tympani nerve at the base of the tongue toward the epithelial surface. Because branching of axons from the chorda tympani is normal in both $nt4^{-/-}$ and $bdnf^{-/-}$

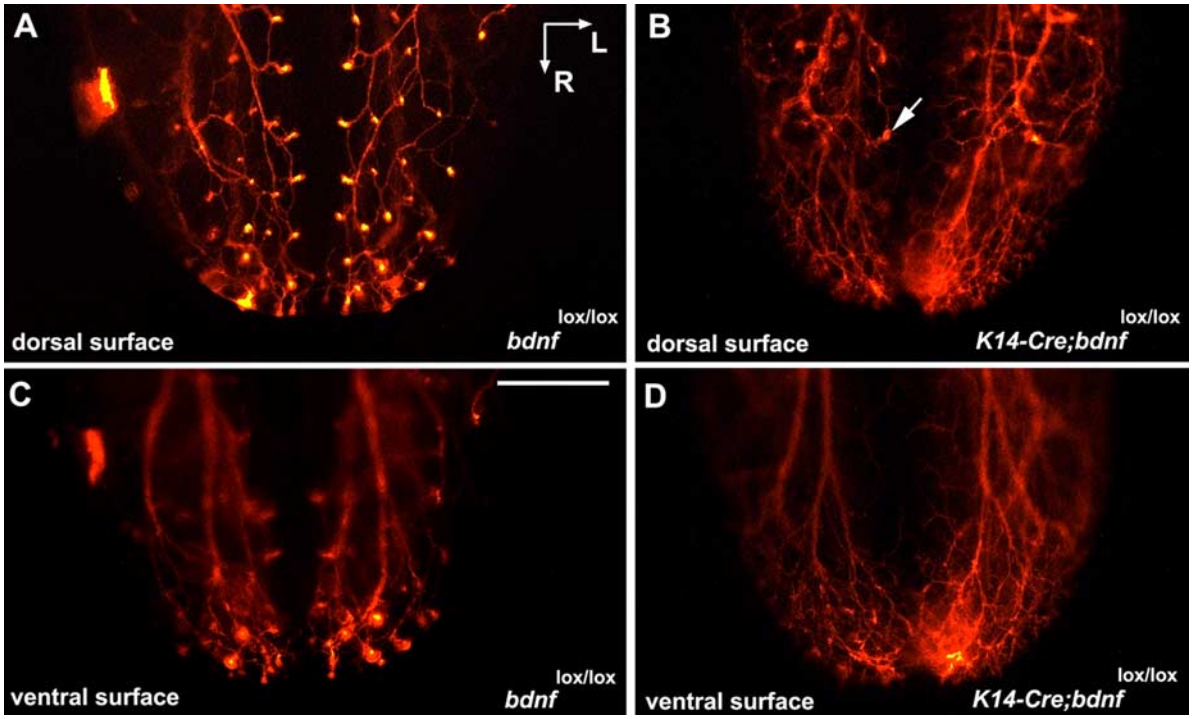

Figure 8. K14-Cre-induced recombination of both *bdnf* alleles disrupts innervation patterns at the tongue tip. ***A***, ***B***, DiI-labeled branching patterns in the dorsal tongue tip at E16.5. ***C***, ***D***, Branching patterns in the ventral tongue tip. E16.5 $bdnf^{lox/lox}$ mice exhibited branching patterns in the dorsal (***A***) and ventral (***C***) tongue tip that were similar to those in wild-type animals (data not shown). Most chorda tympani fiber bundles ended in a clear neural bud near the tongue surface. In *K14-Cre;*$bdnf^{lox/lox}$ mice, branching increased and the pattern of branching at the dorsal (***B***) and ventral (***D***) tongue tip was disrupted. Branching looked more like wild-type branching in caudal regions of the dorsal tongue tip, where some neural buds were evident (***B***, arrow). Scale bar: (in ***C***) ***A–D***, 250 μm.

tongues, but almost absent in $bdnf^{-/-}/nt4^{-/-}$ tongues, we propose that these two factors function interchangeably to support branching toward the lingual surface. Consistent with this idea, both BDNF and NT4 promote geniculate ganglion neurite outgrowth *in vitro* (Rochlin et al., 2000). Furthermore, because the chorda tympani can locate and enter the tongue normally in $nt4^{-/-}/bdnf^{-/-}$ mice, we conclude that these neurotrophins are not required for the location and invasion of the tongue by the chorda tympani. Instead, chemorepellent factors (Rochlin et al., 2000) and a currently unidentified chemoattractant, which is probably not a neurotrophin, are required to attract gustatory axons to the tongue (Gross et al., 2003; Vilbig et al., 2004).

The role of BDNF as a targeting factor is not restricted to the gustatory system. Most BDNF-dependent sensory systems require BDNF for target innervation (Hellard et al., 2004). In these systems, BDNF-dependent fibers reach their target but fail to invade it. However, only gustatory neurons respond to the absence of BDNF with increased branching and innervation of inappropriate targets. Why BDNF removal increases innervation in the gustatory system but not these other systems is unclear. This differential response may be attributable to the fact that only gustatory neurons are also NT4 dependent. In the absence of BDNF, NT4 may function via TrkB to cause hyperinnervation of the tongue. The loss of hyperinnervation to the tongue surface in $bdnf^{-/-}/nt4^{-/-}$ mice is consistent with this possibility. Unlike most BDNF-dependent sensory systems, absence of BDNF can result in hyperinnervation and a failure in axon pruning in the sympathetic ganglion (Kohn et al., 1999; Singh et al., 2008). Thus, unlike for other BDNF-dependent systems, BDNF could have multiple functions in the developing taste system, one of which is axon pruning.

The increased chorda tympani branching in $bdnf^{-/-}$ mice may represent an alternate targeting strategy. During development, gustatory axons could theoretically locate fungiform placodes/papillae by hyperinnervating the tongue and then withdrawing connections from nontaste regions. However, this does not occur during normal gustatory development (Mbiene and Mistretta, 1997). Instead, gustatory fibers are directed to their targets by a molecular factor. Our results show that one molecular factor required for targeting is BDNF. In the absence of functional BDNF, gustatory neurons hyperinnervate the tongue and withdraw from nontaste regions. As a result, some chorda tympani fiber bundles do innervate fungiform papillae by E18.5. Papillae that were innervated between E16.5 and E18.5 in $bdnf^{-/-}$ mice were located at the tongue tip, where papillae are most dense. Consistent with this observation, increased branching of chorda tympani afferent fibers may allow some of these taste afferents to successfully reach fungiform papillae by chance. Alternatively, in the absence of BDNF, some chorda tympani fiber bundles may be directed to the correct target by a later developing targeting factor.

Many taste buds are lost before birth in $bdnf^{-/-}$ mice; however, as many as 43% of fungiform taste buds remain on the tongue at late postnatal ages (Mistretta et al., 1999). Fungiform taste buds are eventually lost in the absence of gustatory innervation (Nagato et al., 1995; Sollars and Hill, 2000; Sollars et al., 2002; Sollars, 2005). Thus, most of the fungiform taste buds remaining in $bdnf^{-/-}$ mice are likely innervated. Because we observed very little specific innervation of fungiform papillae at E14.5, these remaining taste buds are probably located within the fungiform papillae that become innervated between E16.5 and E18.5. Consistent with this possibility, both the later innervated papillae and the remaining taste buds in $bdnf^{-/-}$ mice (Mistretta et al., 1999) are located on the tongue tip. Chorda tympani fiber

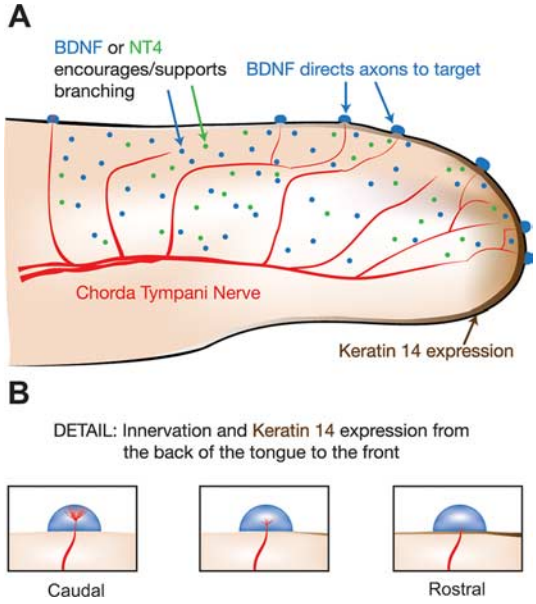

Figure 9. A diagram illustrating the role of neurotrophins in taste bud innervation. During development, the chorda tympani extends in a caudal-to-rostral manner without the support of neurotrophins (***A***). Either BDNF (blue dots) or NT4 (green dots) expressed by the tongue mesenchyme can support chorda tympani branching from the chorda tympani at the base of the tongue. Once fiber branches near the epithelial surface, they are attracted by BDNF-expressing fungiform placodes (blue half-circles). BDNF derived from fungiform papillae attracts chorda tympani fibers to the papilla epithelium and encourages them to penetrate the epithelial surface. Papilla innervation proceeds in a caudal-to-rostral manner, with more caudally located papillae being innervated earlier than more rostrally located papillae (***B***). The timing of epithelial maturation is independent of innervation. Specifically, keratin-14 expression (***A***, brown color) begins at the tongue tip and progresses caudally.

bundles that eventually succeed in locating a fungiform papilla are apparently capable of maintaining taste buds until late postnatal ages.

In conclusion, the findings of the current study are consistent with the following developmental scenario. Early during development, chorda tympani axons enter the tongue independently of neurotrophins. As the chorda tympani nerve extends along the base of the tongue in a caudal-to-rostral manner, fiber bundles branch from the chorda tympani nerve at the tongue base, such that caudal papillae are the first to be innervated (Fig. 9). The timing of this caudal-to-rostral progression of innervation is unrelated to the rostral-to-caudal sequence of Keratin 14 expression (Fig. 9*A*, brown gradient); this timing indicates that lingual epithelium matures in a rostral-to-caudal direction. Interestingly, papillae formation occurs from medial to lateral (Paulson et al., 1985), suggesting that the timing of these three events are unrelated and that some papillae are more mature than others when they are initially innervated. As the chorda tympani enters the tongue, its branches are supported by BDNF and/or NT4. Finally, at E13.5, the chorda tympani branches near the lingual surface, and BDNF expressed by the developing gustatory epithelium directs chorda tympani fibers to invade and innervate fungiform papillae by E14.5.

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