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Postnatal reduction of BDNF regulates the developmental remodeling of taste bud innervation

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ABSTRACT

The refinement of innervation is a common developmental mechanism that serves to increase the specificity of connections following initial innervation. In the peripheral gustatory system, the extent to which innervation is refined and how refinement might be regulated is unclear. The initial innervation of taste buds is controlled by brain-derived neurotrophic factor (BDNF). Following initial innervation, taste receptor cells are added and become newly innervated. The connections between the taste receptor cells and nerve fibers are likely to be specific in order to retain peripheral coding mechanisms. Here, we explored the possibility that the down-regulation of BDNF regulates the refinement of taste bud innervation during postnatal development. An analysis of BDNF expression in $Bdnf^{tlacZ/+}$ mice and real-time reverse transcription polymerase chain reaction (RT-PCR) revealed that BDNF was down-regulated between postnatal day (P) 5 and P10. This reduction in BDNF expression was due to a loss of precursor/progenitor cells that express BDNF, while the expression of BDNF in the subpopulations of taste receptor cells did not change. Gustatory innervation, which was identified by P2X3 immunohistochemistry, was lost around the perimeter where most progenitor/precursor cells are located. In addition, the density of innervation in the taste bud was reduced between P5 and P10, because taste buds increase in size without increasing innervation. This reduction of innervation density was blocked by the overexpression of BDNF in the precursor/progenitor population of taste bud cells. Together these findings indicate that the process of BDNF restriction to a subpopulation of taste receptor cells between P5 and P10, results in a refinement of gustatory innervation. We speculate that this refinement results in an increased specificity of connections between neurons and taste receptor cells during development.

1. Introduction

Neural circuits reorganize throughout development. For example, individual muscle fibers are temporarily innervated by multiple motor axons; the postnatal elimination of axonal branches reduces the number of target cells innervated by each axon (Walsh and Lichtman, 2003). Innervation within the taste bud also may reorganize during development. Developing taste buds are localized to specific epithelial structures called papillae, and taste fibers are directed to these taste buds during initial embryonic targeting (Lopez and Krimm, 2006b; Mbiene and Mistretta, 1997). Remodeling is not required for neurons to make initial connections with taste buds. However, following initial innervation, taste buds continue to grow and differentiate postnatally (Bigiani et al., 2002; Hosley and Oakley, 1987; Kinnamon et al., 2005; Ohtubo et al., 2012; Zhang et al., 2008), resulting in an adult taste bud that has multiple different cell types based on anatomy, function, and expression (Clapp et al., 2006, 2004; Delay et al., 1986; Finger, 2005; Kataoka et al., 2008; Murray and Murray, 1967; Murray et al., 1969; Yang et al., 2000a, 2000b; Yee et al., 2001). Therefore, it would not be surprising if some developmental remodeling were required for taste receptor cells to be innervated by the appropriate nerve fibers. Consistent with this idea, taste innervation is reduced postnatally in the rat and sheep in a manner that suggests reorganization (Kinnamon et al., 2005; Mistretta et al., 1988; Nagai et al., 1988).

Although it is well established that synaptic reorganization can be driven by afferent activity (Erzurumlu and Kind, 2001; Espinosa and Stryker, 2012; Kirkby et al., 2013), developmental factors that regulate survival, axon guidance, and targeting can also regulate postnatal synaptic reorganization and the specificity of connections (Gonzalez et al., 1999; Nguyen et al., 1998; Pfeiffenberger et al., 2005). For example, the neurotrophin, brain-derived neurotrophic factor (BDNF), regulates the critical period in which eye-specific input is segregated in the visual cortex (Cabelli et al., 1997;

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Huang et al., 1999). BDNF also is an important guidance factor for peripheral taste neurons that allows taste afferents to find and innervate taste buds during embryonic development (Lopez and Krimm, 2006a; Ma et al., 2009). Interestingly, this process occurs during a critical period, after which BDNF is no longer required to innervate a taste bud (Ma et al., 2009). Yet, BDNF is still expressed in postnatal and adult taste buds (Huang and Krimm, 2010; Yee et al., 2003). Therefore, as with other systems BDNF could have a role remodeling innervation within taste buds during postnatal development.

Our goal was to examine the role of BDNF during postnatal development. During embryonic development, the expression patterns of BDNF in the peripheral taste system correlate with their roles in neuronal survival and target innervation (Huang and Krimm, 2010). Therefore, we wanted to determine when BDNF is expressed, and in which types of taste cells during various stages of postnatal development. We also wanted to determine the potential involvement of BDNF in the postnatal remodeling of innervation within the taste bud. We found that a reduction of BDNF in progenitor/precursor cells between postnatal day 5 and 10 mediates the postnatal refinement of innervation within the taste bud.

2. Materials and methods

2.1. Animals

Bdnf$^{LacZ/+}$ mice, in which the *Bdnf* coding sequence at one allele is replaced by the *E. coli* galactosidase *(LacZ)* sequence, were used to determine the localization of BDNF expression (Jones et al., 1994). *K14-Bdnf*-OE mice, in which the expression of BDNF is driven by the keratin-14 promoter, were used to overexpress BDNF in the tongue epithelial progenitor cells, including those of the taste buds (Krimm et al., 2001; LeMaster et al., 1999; Lopez and Krimm, 2006a). Mice were analyzed at birth and postnatal days (P) 5, 10, 20, and 60 (adult). All animals were cared for and studied in accordance with the guidelines set by the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Laser capture microdissection and RNA extraction

Taste buds and geniculate ganglion cells were isolated from newborn and postnatal mice using laser capture microdissection (LCM) using the protocols described previously (Huang and Krimm, 2010). Briefly, the anterior tongue and circumvallate (CV) were dissected and then sectioned (10-μm) and processed to visualize the taste buds. The identified taste buds were captured onto CapSure Macro LCM Caps (Molecular Devices, Sunnyvale, CA, USA). For each animal, all captured samples were stored for RNA isolation.

Total RNA was extracted from the taste buds using an RNeasy micro kit according to the manufacturer's instructions (#74004; Qiagen, Germantown, MD, USA). DNase I treatment was applied to eliminate traces of DNA during the procedure. Following isolation, the RNA quality was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA Integrity Number (RIN) and 28S/18S ratio were used to estimate the RNA quality. Only RNA samples with a 260/280 ratio $\geq$ 1.80 and RIN $\geq$ 8.0 were used.

2.3. Real-time reverse transcription polymerase chain reaction

Taste bud cDNA was synthesized from total RNA using random primers (Invitrogen, Carlsbad, CA, USA). The cDNA was quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) using a TaqMan Universal PCR kit (#4304437; Applied Biosystems, Waltham, MA, USA). The real-time RT-PCR reactions were conducted using 10 μl total volume with 300 nM primers. We used the same primers reported in a previous study (Huang and Krimm, 2010). For the normalization of the cDNA loading, all samples were run in parallel with the 18 S ribosomal RNA housekeeping gene. Real-time RT-PCR was performed with ABI PRISM/7900HT Sequence detection systems (Applied Biosystems). Each assay was conducted in triplicate. The RT-PCR conditions were an initial incubation of 50 °C for 2 min and 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.

2.4. β-galactosidase (X-Gal) staining

To detect β-galactosidase, X-Gal staining was performed on the tissues dissected from *Bdfn*$^{LacZ/+}$ mice using procedures described in a previous study (Huang and Krimm, 2010). Briefly, newborn and postnatal mice were perfused and post-fixed with 0.5% glutaraldehyde, and rinsed in PBS/$MgCl_2$. The anterior tongue, CV, and geniculate ganglia were dissected and frozen in OCT. These tissues were sectioned (16 μm) and stained using β-galactosidase (β-Gal) staining solution (InvivoGen, San Diego, CA, USA). Images of the β-Gal staining in the taste buds and geniculate ganglion were taken using a Retiga 1300 digital camera (QImaging, Surrey, BC, Canada) mounted to a DMLB Leica microscope.

2.5. Immunohistochemistry

Newborn and postnatal mice were perfused with 4% paraformaldehyde. The anterior tongue and CV were dissected and post-fixed overnight, cryoprotected in 30% sucrose, and frozen in OCT. For immunohistochemistry, the anterior tongue was cut at a thickness of 50 μm, and the sections were collected into 0.1 M PB and rinsed. After blocking with 3% normal serum in 0.1 M PB containing 0.5% Triton X-100, the tissues were incubated with primary antibodies (Table 1) for 5–7 days at 4 °C. Monovalent Fab fragment antibody (#711-007-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used in multiple labeling experiments when the primary antibodies were from the same species (rabbit). After the samples were incubated with primary antibodies and rinsed, appropriate secondary antibodies (Jackson ImmunoResearch Laboratories), including Alexa Fluor 488 (#711-545-152; #705-545-147; #712-545-153), Alexa Fluor 647 (#712-605-153), and Cyanine Cy3 (#711-165-152), were applied overnight. The tissues then were washed and mounted with aqueous mounting medium (Fluoromount-G; SouthernBiotech, Birmingham, AL, USA). Serial optical sections were captured every 1 μm in labeled whole taste buds using a confocal microscope (FV1200; Olympus) under a 60 × lens at 3.5 zoom. Each label was collected separately with single wavelength excitation and then merged to produce the composite image. At least 5 (for taste innervation) or 10 (for the co-expression of BDNF with taste bud cell markers) taste buds from each animal were captured for further analyses.

Table 1
The antibodies used in the study.

Antibody	Host	Concentration	Company	Cat. #
5-HT	Rabbit	1:500	ImmunoStar	20080
β-Gal	Rabbit	1:1000	MP biomedicals	55976
Car4	Goat	1:500	R&D systems	AF2414
Keratin-8	Rat	1:50	Developmental studies hybridoma bank	Troma-1
NTPDase2	Rabbit	1:500	CHUL	mN2-35 L; mN2-36 L
P2X3	Rabbit	1:500	Millipore	AB5895
PCLβ2	Rabbit	1:500	Santa Cruz	Sc-206
SNAP25	Rabbit	1:500	Millipore	AB1762