Molecular and Cellular Neuroscience 82 (2017) 195–203

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Taste bud-derived BDNF maintains innervation of a subset of TrkB-expressing gustatory nerve fibers

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ARTICLE INFO

Article history:
Received 16 January 2017
Revised 8 May 2017
Accepted 5 June 2017
Available online 20 June 2017

Keywords:
Taste
Taste bud innervation
Neurotrophins
BDNF
Sensory
TrkB

ABSTRACT

Taste receptor cells transduce different types of taste stimuli and transmit this information to gustatory neurons that carry it to the brain. Taste receptor cells turn over continuously in adulthood, requiring constant new innervation from nerve fibers. Therefore, the maintenance of innervation to taste buds is an active process mediated by many factors, including brain-derived neurotrophic factor (BDNF). Specifically, 40% of taste bud innervation is lost when *Bdnf* is removed during adulthood. Here we speculated that not all gustatory nerve fibers express the BDNF receptor, TrkB, resulting in subsets of neurons that vary in their response to BDNF. However, it is also possible that the partial loss of innervation occurred because the *Bdnf* gene was not effectively removed. To test these possibilities, we first determined that not all gustatory nerve fibers express the TrkB receptor in adult mice. We then verified the efficiency of *Bdnf* removal specifically in taste buds of *K14-CreER*:*Bdnf* mice and found that *Bdnf* expression was reduced to 1%, indicating efficient *Bdnf* gene recombination. BDNF removal resulted in a 55% loss of TrkB-expressing nerve fibers, which was greater than the loss of P2X3-positive fibers (39%), likely because taste buds were innervated by P2X3 + /TrkB − fibers that were unaffected by BDNF removal. We conclude that gustatory innervation consists of both TrkB-positive and TrkB-negative taste fibers and that BDNF is specifically important for maintaining TrkB-positive innervation to taste buds. In addition, although taste bud size was not affected by inducible *Bdnf* removal, the expression of the γ subunit of the ENaC channel was reduced. So, BDNF may regulate expression of some molecular components of taste transduction pathways.

1. Introduction

In the tongue, clusters of taste receptor cells organized into taste buds respond to chemicals in food. Taste buds are innervated by neurons of the geniculate ganglion, which carry taste information to the brain. Taste receptor cells have a limited lifespan and continuously turnover (Beidler and Smallman, 1965; Perea-Martinez et al., 2013). As taste receptor cells die, functional connections between taste receptor cells and nerve fibers are lost. When new taste receptor cells enter taste buds, they must become innervated by nerve fibers and form new connections with gustatory neurons. Thus, the maintenance of innervation within taste buds is an active process that likely depends on many molecular mechanisms.

Clues as to the nature of these mechanisms could come from similar developmental processes. In particular, brain-derived neurotrophic factor (BDNF) directs the innervation of newly formed taste buds during development (Krimm et al., 2001; Lopez and Krimm, 2006; Ma et al., 2009; Ringstedt et al., 1999). As BDNF is also expressed in adult taste buds (Huang et al., 2015; Yee et al., 2003), it could continue to maintain taste bud innervation throughout the taste cell's lifespan. Indeed, taste bud innervation is lost when the *Bdnf* gene is removed from adult animals (Meng et al., 2015). Interestingly, however, considerable innervation to taste buds remains after BDNF removal.

One possible explanation for this remaining innervation is that there are different "types" of gustatory neurons that vary in their BDNF dependence due to differences in receptor expression. By binding to TrkB receptors, BDNF initiates multiple signaling pathways that regulate neuronal survival, synaptic plasticity, and differentiation (Minichiello, 2009; Numakawa et al., 2010; Waterhouse and Xu, 2009). If not all nerve fibers express TrkB receptors, BDNF removal may only affect neurons that express this receptor. Consistent with this idea, only some geniculate ganglion neurons express TrkB receptors (Cho and Farbman, 1999; Farbman et al., 2004a, 2004b; Fei and Krimm, 2013; Yamout et al., 2005). A second possible explanation is that the genetic construct (keratin 14 (K14)-CreER) used in this earlier study (Meng et al., 2015) does not completely remove BDNF from all taste receptors cells, as some cells could be derived from connective tissue cells that lack K14 (Boggs et al., 2016).

The goal of the current study was to distinguish between these two possibilities. First, we aimed to verify efficient *Bdnf* removal from taste buds and to determine whether TrkB is expressed in a subset of

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http://dx.doi.org/10.1016/j.mcn.2017.06.001
1044-7431/

innervating taste fibers in adult mice. Next, we examined whether TrkB-positive fibers are impacted by *Bdnf* removal from the epithelium and taste buds. We also sought to determine whether taste bud-derived BDNF plays a role in maintaining taste bud size or taste receptor expression, either of which could influence taste function. We found that *Bdnf* expression was efficiently eliminated from taste buds in K14-CreER mice. Furthermore, BDNF supported a large subset of TrkB-expressing nerve fibers and not TrkB-negative nerve fibers. Lastly, BDNF maintained expression of γ subunit of ENaC in taste buds. Thus, BDNF maintains taste bud innervation in adulthood of a particular subset of nerve fibers and regulates factors required for taste transduction.

2. Materials and methods

2.1. Animals

Inducible *Bdnf* mutants were produced by breeding mice with floxed *Bdnf* alleles ($Bdnf^{flox/+}$; Jackson Laboratory, 002267) with mice with an inducible Cre-mediated recombination system driven by a K14 promoter (K14-CreER; #005107, Jackson Laboratory). Gene recombination under control of the K14 promoter results in successful gene recombination in cells that differentiate into taste buds (Okubo et al., 2009; Vasioukhin et al., 1999). These mice were bred with heterozygous $Bdnf^{+/-}$ knock-out mice (#002266, Jackson Laboratory) and mice in which a green fluorescent protein (GFP) cassette was inserted into the first coding exon of Ntrk2 ($TrkB^{GFP/+}$) to visualize TrkB-expressing nerve fibers (Li et al., 2011). Thus, mice used for anatomical analysis lacked a functional *Bdnf* gene in one allele, *Bdnf* could be inducibly removed from the other allele and GFP is expressed in TrkB-positive neurons (*K14-CreER:*$Bdnf^{flox/-}$:$TrkB^{GFP/+}$). Three control genotypes were used for different purposes of comparison. $Bdnf^{flox/+}$:$TrkB^{GFP/+}$ mice (with tamoxifen) were used to exclude any effects of tamoxifen administration, and *K14-CreER:*$Bdnf^{flox/+}$:$TrkB^{GFP/+}$ mice (without tamoxifen) were used to exclude the possibility of gene recombination in the absence of tamoxifen; both genotypes were expected to produce wild-type levels of BDNF. $Bdnf^{flox/-}$:$TrkB^{GFP/+}$ mice (with tamoxifen) were used to control for any effects of heterozygous *Bdnf* knock out. In addition, we bred K14-CreER and K14-Cre mice with mice expressing tdTomato (#007914, Jackson Laboratory) to visualize the effectiveness of tamoxifen-induced gene recombination. To measure *Bdnf* gene expression by real-time reverse transcription polymerase chain reaction (RT-PCR), the same genotypes without $TrkB^{GFP/+}$ were used.

2.2. Tamoxifen administration

Mice received tamoxifen (T5648, Sigma-Aldrich, St. Louis, MO; mixed in peanut oil, 188 ng/g body weight) once per day for 3 weeks by oral gavage. This dose was previously used for effective inducible gene recombination in adult mice (McGraw et al., 2011; Meng et al., 2015; Ruzankina et al., 2007). Tamoxifen administrations were initiated in all mice around 60 days of age. Mice were euthanized 10 weeks after the final tamoxifen administration.

2.3. Laser capture microdissection, RNA extraction, and cDNA amplification and purification

Mice were euthanized by an overdose of 2.5% tribromoethanol (Avertin) i.p. (T48402, Sigma-Aldrich, St. Louis, MO; mixed in tert-amyl alcohol, then diluted in 1/40 PBS). Taste buds were isolated using laser capture microdissection (LCM) as previously described (Huang and Krimm, 2010). The anterior part of the tongue was removed, rinsed with 0.1 M PBS solution (pH 7.4), and cut in half under a microscope. Each half was placed into a disposable embedding mold, covered with OCT, and frozen immediately and stored at −80 °C for future use. Identified taste buds were captured onto CapSure Macro LCM Caps (Molecular Devices, Sunnyvale, CA). For each mouse, all captured samples were stored for RNA isolation.

Total RNA was extracted from taste buds using an RNeasy micro kit according to the manufacturer's instructions (#74004, Qiagen, Germantown, MD). DNase I treatment was applied to eliminate traces of DNA during the procedure. Following isolation, RNA quality was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and estimated by the RNA Integrity Number (RIN) and 28S/18S ratio. Only RNA samples with a 260/280 ratio ≥ 1.80 and RIN ≥ 8.0 were used. Taste bud cDNA was synthesized from total RNA using random primers (Invitrogen, Carlsbad, CA).

2.4. Real-time RT-PCR

Real-time RT-PCR was performed using ABI PRISM/7900HT Sequence detection systems (Applied Biosystems, Waltham, MA, USA) with TaqMan Universal PCR kits (#4304437, Applied Biosystems) and oligonucleotide primer/probe sets, which were designed from sequences available in the GenBank Database using Beacon Designer software (Premier Biosoft International, Atlanta, GA). When possible, primers were chosen to span an intron to avoid genomic DNA contamination (Table 1). TaqMan probes were labeled at the 5′ end with a fluorescent dye (fluorescein) and at the 3′ end with a quencher dye (carboxytetramethylrhodamine). Real-time RT-PCR reactions were conducted using 10 μl 1× Master Mix, 720/200 nm primer/probe sets (TaqMan PCR kit), and the same amount of target cDNA. For normalization of cDNA loading, all samples were run in parallel with 18S ribosomal RNA, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and cytokeratin 8 (K8). Each assay was carried out in triplicate. Amplification of cDNA was performed for 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.5. Immunohistochemistry

Mice were euthanized by an overdose (1 ml) of 2.5% of Avertin, transcardially perfused with 4% paraformaldehyde (PFA), and post-fixed in 4% PFA for 2 h or immersion-fixed in 4% PFA overnight. The anterior part of the tongue was dissected and post-fixed overnight in 4% PFA. The tissue was transferred to 30% sucrose at 4 °C overnight, frozen in OCT, and stored at −80 °C until sectioned on a cryostat. To visualize whole taste buds and their innervation, tongues were sectioned at 70 μm, and the sections were collected in 0.1 M phosphate-buffered saline (PBS) and rinsed four times in PBS. Cryostat sections were blocked with 3% normal donkey serum in 0.1 M PBS containing 0.5% Triton X-100. The tissue was incubated with the following primary antibodies for 5 days at 4 °C: rat anti-K8 in PBS (1:50; Developmental Studies Hybridoma Bank, AB Registry ID: AB_531826, cat#: Troma-1-s, Iowa City, IA), goat anti-GFP (1:400; Novus, AB Registry ID: AB_10128178, cat#: NB100-1700, Littleton, CO), or rabbit anti-P2X3 (1:500; Millipore, AB Registry ID: AB_11212062, cat#: AB58950, Billerica, MA). After incubation in primary antibodies and four rinses in PBS, sections were incubated in the following secondary antibodies for 2 days: anti-rat Alexa Fluor 555 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA), anti-goat Alexa Fluor 488 (1:500; Jackson ImmunoResearch Laboratories), or anti-rabbit Alexa Fluor 647 (1:500; Jackson ImmunoResearch Laboratories). The tissue was then washed four times in 0.1 M PBS, mounted onto slides, and cover-slipped using aqueous mounting medium (Fluoromount-G, SouthernBiotech, Birmingham, AL).

2.6. Quantification of taste bud innervation and volume

Taste buds from the tip of the tongue (front 1/3 of fungiform field) were imaged using a confocal microscope (Olympus, model no. FV1200BX61). During both image capture and analysis, the experimenter was blind to mouse genotype. Optical images were captured every 1 μm with a 60× oil immersion lens at a zoom level of 3.5 from the front one-third of the fungiform field (i.e., tongue tip). For each image, all