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

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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.

Keywords

Taste; Taste bud innervation; Neurotrophins; BDNF; Sensory; TrkB

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

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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 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 ($Bdnf^{lox/+}$; 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*$^{lox/-}$:*TrkB*$^{GFP/+}$). Three control genotypes were used for different purposes of comparison. *Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$ mice (with tamoxifen) were used to exclude any effects of tamoxifen administration, and *K14-CreER:Bdnf*$^{lox/+}$:*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*$^{lox/-}$:*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, German-town, 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 de-hydrogenase (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 three channels were taken separately using single-wavelength excitation and merged to produce a composite image. Imaris software (Bitplane, http://www.bitplane.com/contact) was used to rotate taste buds

and determine whether the whole taste bud was captured. Innervation density in the first six whole taste buds imaged per mouse tongue was determined. For each optical section, taste buds were outlined using K8 labeling to define the perimeter. Next, the total taste bud volume, the volume occupied by the 647 label (representative of P2X3-labeled nerve fibers), and the volume occupied by the 488 label (representative of TrkB-labeled nerve fibers) were measured separately using the surface feature in Imaris. Because the TrkB-GFP construct labels both full-length and truncated-TrkB, there is truncated TrkB in the taste bud and surround tissues. Taste fibers express TrkB-GFP at much higher levels than surrounding tissues. Therefore, imaging software was used to set a threshold for labeling that included the nerve fibers and not the taste buds.

2.7. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). For real-time RT-PCR results, the comparative $2^{-\Delta\Delta CT}$ method was used to quantify target gene expression (Livak and Schmittgen, 2001). The Shapiro-Wilk test was used to assess whether data were normally distributed. One-way analysis of variance (ANOVA) was used to test for differences between genotypes in mRNA levels, taste bud volume, and nerve fiber innervation. Two-way ANOVA was used to test for differences between genotypes in P2X3- and TrkB-labeled fibers. After significant overall ANOVAs, Student-Newman-Keuls post-hoc tests were used for pairwise comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Taste bud innervation can be divided into TrkB-positive and TrkB-negative fibers

Although taste bud innervation is reduced when the *Bdnf* gene is removed from adult mice (Meng et al., 2015), a surprising amount of innervation remains. One possible explanation is that some fibers innervating taste buds are not responsive to BDNF because they do not express TrkB receptors. Therefore, we examined whether all taste fibers express the high-affinity BDNF receptor, TrkB, by labeling taste buds with anti-P2X3 (blue) and anti-GFP (green) in $TrkB^{GFP/+}$ mice (Fig. 1A–D). P2X3 is an ATP channel required for neuronal responses to taste stimuli (Finger et al., 2005), and its antibody labels taste fibers within taste buds. Anti-K8 was used to label taste buds (red). We found that many fibers were labeled with both anti-P2X3 and anti-GFP. However, some P2X3-positive fibers were not labeled with anti-GFP (arrows in Fig. 1A–C). Therefore, some P2X3-positive fibers did not express TrkB. We also observed more weakly GFP-labeled perigemmal cells. We suspect that these cells express a truncated form of the TrkB receptor, which is expressed in most epithelia and can be used to sequester BDNF to a particular location (Liu et al., 2012).

The cell bodies for the nerve fibers that innervate fungiform taste buds are located in the geniculate ganglion. To determine whether all geniculate ganglion neurons express TrkB, we double-labeled the geniculate ganglion from $TrkB^{GFP/+}$ mice with anti-GFP and anti-P2X3 (Fig. 1E–G) and determined the percentage of double-labeled cells. We found that 56 ± 7% of P2X3-positive neurons also expressed GFP. Together, these data indicate that not all gustatory neurons express TrkB during adulthood. Therefore, some nerve fibers may remain in taste buds after BDNF removal because they are not responsive to BDNF.

3.2. BDNF was effectively and specifically removed from taste buds under the control of the K14 promoter

To further examine why taste bud innervation remains after *Bdnf* removal, we re-evaluated the effectiveness of *Bdnf* removal from taste buds in K14-CreER mice. Although previous reports indicate that all types of taste bud cells arise from K14+/K5+ basal cells (Gaillard et al., 2015; Okubo et al., 2009), a recent study shows that some taste bud cells may migrate from connective tissue (Boggs et al., 2016). To examine if most taste bud cells appear to originate from K14+ progenitor cells, we bred K14-Cre mice with stop-floxed tdTomato (Ai14) mice ($n = 2$). In these mice, the lingual epithelium and taste buds were brightly stained with tdTomato (red, Fig. 2A). Moreover, all fungiform taste buds examined were completely labeled with tdTomato (Fig. 2B), indicating that all taste bud cells were derived from cells that at some point expressed K14. We wanted to verify that taste receptor cell types that express BDNF (Car4+) undergo gene recombination. Taste bud cells labeled with anti-carbonic anhydrase 4 (Car4, blue), which also expresses *Bdnf* (Huang et al., 2015), were also labeled with tdTomato (Fig. 2C–D), indicating that they originated from K14+ progenitor cells. To visualize the effectiveness of *K14-CreER*-induced gene recombination, we bred *K14-CreER* mice with stop-floxed tdTomato (Ai14) mice. Ten weeks after the end of tamoxifen administration (3 weeks), the fungiform papillae and taste buds were labeled with tdTomato (Fig. 2E, $n = 3$), and most taste bud cells appeared to be derived from cells that underwent gene recombination (red, Fig. 2F). Many taste bud cells were double-labeled with anti-Car4 and tdTomato (arrow, Fig. 2F–H), indicating that taste bud cells that expressed *Bdnf* underwent gene recombination in K14-CreER mice.

A previous study shows that *Bdnf* expression was decreased in the whole epithelium after 3 weeks of tamoxifen administration in *K14-CreER:Bdnf*$^{lox/-}$ mice (Meng et al., 2015); however, *Bdnf* expression in taste buds was not specifically examined. One possible explanation for why innervation remains in taste buds after *Bdnf* gene recombination is that BDNF is more effectively removed from the epithelium than from taste buds. To assess whether *Bdnf* gene recombination changes *Bdnf* expression levels in taste buds, we isolated taste buds using LCM and performed real-time RT-PCR. We found that *Bdnf* was reduced to <1% in *K14-CreER:Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$ mice ($n = 4$) compared with three control genotypes (*Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.01$, ($n = 4$); *K14-CreER:Bdnf*-$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.05$, ($n = 4$); *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, $p < 0.05$, ($n = 4$); Fig. 2I). This reduction was greater than previously observed in the whole epithelium (Meng et al., 2015). Therefore, it does not appear that nerve fibers remain because of inefficient gene recombination in taste buds.

3.3. BDNF regulates TrkB-positive but not TrkB-negative innervation within taste buds

After demonstrating that remaining taste bud innervation is not due to inadequate *Bdnf* gene removal, we speculated that TrkB-negative fibers may not depend on BDNF during adulthood. To address this issue, we quantified TrkB-positive innervation after *Bdnf* removal from taste buds by breeding mice in which *Bdnf* could be inducibly removed by tamoxifen with mice in which TrkB-positive fibers were genetically labeled (Li et al., 2011). Taste fibers were labeled with anti-P2X3 (blue, most gustatory innervation) and anti-GFP (green, TrkB-positive innervation), and taste buds were labeled with anti-K8 (red). All three control genotypes (*Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, ($n = 4$), *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, ($n = 4$),

Bdnf$^{lox/-}$:*TrkB*$^{GFP/+}$, (n = 4), Fig. 3A–L) and mice in which *Bdnf* expression was reduced (*K14-CreER:Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, (n = 4), Fig. 3M–P) had P2X3- and GFP-labeled innervation within taste buds. However, taste buds appeared to have less P2X3- and GFP-labeled innervation in mice with reduced *Bdnf* (*K14-CreER: Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$) compared to control genotypes. No obvious difference in taste bud size was observed across genotypes.

To quantify the effect of *Bdnf* gene removal on total innervation, we analyzed the volume of P2X3-positive and TrkB-positive innervation within taste buds. Ten weeks after tamoxifen administration, the volume of remaining P2X3-positive innervation differed among genotypes ($F_{(3,12)}$ = 10.8, $p < 0.001$). Specifically, taste buds in *K14-CreER:Bdnf*-$^{lox/-}$:*TrkB*$^{GFP/+}$ mice showed 39% less P2X3-positive innervation than the three control genotypes (*Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.005$; *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.01$; *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, $p < 0.005$; Fig. 4A). Similarly, the volume of TrkB-positive innervation also differed among genotypes ($F_{(3,12)}$ = 35.4, $p < 0.001$). *K14-CreER:Bdnf*$^{lox/-}$:*TrkB*-$^{GFP/+}$ mice showed 55% less TrkB-positive innervation than the three control genotypes (*Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.001$; *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.001$; *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, $p < 0.001$; Fig. 4A). There were no differences in the volume of P2X3-positive or TrkB-positive innervation among the three control genotypes, indicating that neither tamoxifen nor elimination of a single *Bdnf* allele altered the amount of innervation within taste buds. Furthermore, removal of the *Bdnf* gene from the epithelium and taste buds caused a greater loss of TrkB-positive innervation than total (i.e., P2X3-positive) innervation ($p < 0.001$), suggesting that *Bdnf* gene removal primarily affects TrkB-positive fibers.

Using P2X3 as a marker for all gustatory innervation, we estimated the amount of TrkB-negative gustatory innervation by subtracting the amount of TrkB-positive innervation from P2X3-positive innervation for each taste bud. We found no difference in the volume of TrkB-negative innervation among the four genotypes ($F_{(3,12)}$ = 0.74, p = 0.546, Fig. 4B), suggesting that TrkB-negative innervation was not affected by removal of the *Bdnf* gene.

Previously, taste bud volume was reduced when the *Bdnf* gene was removed from all tissue with a ubiquitous promoter but not when it was specifically removed from taste buds of adult mice (Meng et al., 2015). To confirm these findings, we quantified taste bud volume in *Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$ (n = 4), *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$ (n = 4), *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$ (n = 4) and *K14-CreER:Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$ (n = 4) mice. We found no genotype differences in taste bud volume ($F_{(3,12)}$ = 1.54, p = 0.26, Fig. 4C), indicating that taste bud size is no affected when *Bdnf* is inducibly removed from taste buds.

In summary, we found that the reduced taste bud innervation in mice lacking *Bdnf* in the epithelium was due to a loss of TrkB-positive innervation and not TrkB-negative innervation. However, in addition to the TrkB-negative innervation, a surprising amount of TrkB-positive innervation remained in taste buds. Taste bud size was not affected by *Bdnf* gene removal or loss of innervation.

3.4. BDNF removal reduced expression of the γ subunit of the ENaC receptor

Finally, we investigated whether BDNF signaling has a direct effect on the presence of transduction mechanisms in adult taste buds. Deactivation of specific TrkB signaling

pathways (SHC and PLCγ) during development alters the expression of multiple taste transduction mechanisms in the whole tongue epithelium (Koudelka et al., 2014). However, it is not clear whether this is strictly a developmental role or whether BDNF-TrkB signaling regulates transduction mechanisms in adulthood. Therefore, we used RT-PCR to examine the expression of different taste receptors/channels that transduce taste stimuli in fungiform taste buds of *K14-CreER:Bdnf*$^{lox/-}$ mice and three control genotypes. Salt taste on the front of the tongue is largely transduced by the amiloride-sensitive channel ENaC, which has three subunits. We found that the expression of the γ subunit of ENaC (Scnn1g) was significantly decreased in *K14-CreER:Bdnf*-lox/$^{-}$ mice (Fig. 5A), whereas the expression of β and α subunits did not change (Fig. 5B–C). We observed no changes in the other transduction mechanisms examined. Specifically, there were no differences in the expression of T1R1, T1R2, or T2R5 between *K14-CreER:Bdnf*$^{lox/-}$ mice and the three control genotypes (T1R1, $n = 4$, $p = 0.9713$; T1R2, $n = 4$, $p = 0.8796$; T2R5, $n = 4$, $p = 0.2862$), whereas the sour taste channel PKD2L1 was consistently below the detection threshold. These findings suggest that although the impact of BDNF-TrkB signaling on taste transduction mechanisms may be largely developmental (Koudelka et al., 2014), salt taste transduction could be altered in the taste buds of adult *K14-CreER:Bdnf*$^{lox/-}$ mice.

4. Discussion

Taste bud cells die and turnover continuously in adulthood, and new taste bud cells must be innervated by nerve fibers (Beidler and Smallman, 1965; Perea-Martinez et al., 2013). Thus, the maintenance of innervation to taste buds in adulthood is an active process that is likely regulated by multiple factors. In the absence of BDNF, taste bud innervation is reduced by 40% (Meng et al., 2015), indicating that BDNF is required to maintain some innervation to taste buds. However, 60% of innervation remains in taste buds after *Bdnf* gene removal. We speculated that a subset of taste nerve fibers expressing the BDNF receptor TrkB may be lost by adulthood, thus reducing taste bud innervation, which would be consistent with previous studies demonstrating that some adult gustatory neurons do not express the TrkB receptor (Cho and Farbman, 1999; Farbman et al., 2004b; Matsumoto et al., 2001). We found that some taste nerve fibers do not express the TrkB receptor, and more TrkB-positive innervation than total innervation was lost from adult taste buds after inducible *Bdnf* removal. Therefore, we conclude that taste bud-derived BDNF is specifically required to maintain a subset of TrkB-positive fiber innervation to adult taste buds.

Our results confirm and extend the findings of an earlier study examining BDNF support of gustatory innervation (Meng et al., 2015). BDNF expression was previously measured in the whole lingual epithelium, including both epithelial cells and taste bud cells, making it unclear how much of the 80% reduction in *Bdnf* was specific to taste buds. This is particularly relevant because it is possible that not all taste bud cell precursors express K14 (Boggs et al., 2016), which could result in *Bdnf* remaining in taste buds. We found no taste receptor cells that failed to undergo gene recombination in *K14-CreER:tdTomato* mice, and only a few taste bud cells that failed to undergo gene recombination in adult *K14-CreER:tdTomato* mice. Moreover, *Bdnf* mRNA level was reduced to <1% in taste buds of *K14-CreER:Bdnf*$^{lox/-}$ mice compared to those of three control genotypes. This indicates that most *Bdnf*-expressing cells in taste buds successfully underwent gene recombination, and

only a small proportion of taste bud cells continued to express *Bdnf*. It should be noted that effective gene recombination did require 3 weeks of tamoxifen injections. This is consistent with previous studies demonstrating gene recombination in some but not all basal layer keratinocytes of K14-CreER mice after one week of tamoxifen (Okubo et al., 2009). In addition, the effectiveness of tamoxifen induced gene recombination is likely influenced by multiple factors including the level of expression and location of the floxed allele in the nucleus. BDNF is released in higher levels from remaining cells when the number of BDNF-expressing cells is reduced (Meng et al., 2017), likely requiring almost complete removal for an observable reduction in BDNF. Therefore, tamoxifen dose must be tailored for each experiment, and in this case, 3 weeks of tamoxifen administration was required. However, because we did observe effective gene recombination with tamoxifen, it is unlikely that innervation remains in the taste buds after *Bdnf* removal because *Bdnf*-induced gene recombination was not effective.

We found that some nerve fibers were TrkB-negative and not affected by *Bdnf* removal. Consistently, BDNF is expressed in some adult taste receptor cells but not others (Huang et al., 2015; Yee et al., 2003). This indicates that BDNF only maintains a subset of the total nerve fibers that innervate taste buds, perhaps specifically those nerve fibers that innervate BDNF-expressing taste bud cells. Therefore, in addition to TrkB, another mechanism likely exists to support TrkB-negative innervation. One possibility is that this role is served by another growth factor. For instance, glial cell line-derived neurotrophic factor is expressed in a different subset of taste bud cells than BDNF (Suzuki et al., 2007; Takeda et al., 2004), and its receptors are expressed in the geniculate ganglion (Farbman et al., 2004b; Yamout et al., 2005). However, many other factors could also play this role/s (Feng et al., 2014; Germana et al., 2006; McLaughlin, 2000; Suzuki et al., 2007; Suzuki et al., 2005; Takeda et al., 2005; Yee et al., 2005).

Although TrkB-positive innervation is dramatically reduced by inducible *Bdnf* removal from the epithelium, 45% of TrkB-positive innervation remained in the taste buds. Because the other TrkB-ligand (NT4) is down-regulated in the taste bud during development to undetectable levels by adulthood (Huang and Krimm, 2010), it is unlikely that NT4 is supporting the remaining innervation. There are at least two other possible explanations for the remaining innervation. First, not all BDNF-expressing cells may undergo gene recombination, thus some BDNF protein could still remain to support TrkB-positive nerve fibers. However, given the small remaining amount of *Bdnf* expression, this explanation seems unlikely to account for all of the remaining TrkB innervation. Second, some TrkB-positive nerve fibers may not express an isoform for TrkB allowing BDNF to signal; therefore, BDNF may not support the maintenance of these nerve fibers, even though they express TrkB. The TrkB receptor consists of two isoforms: a full-length and a truncated TrkB receptor (Klein et al., 1990, 1989; Middlemas et al., 1991). Because the truncated isoform of the TrkB receptor is expressed in the geniculate ganglion (Farbman et al., 2004b; Yamout et al., 2005), it is possible that some TrkB-positive nerve fibers only express this isoform. The truncated isoform signals in some instances (Cheng et al., 2007; Islam et al., 2009; Ohira et al., 2006, 2005; Yacoubian and Lo, 2000) but can also function as a dominant-negative receptor that inhibits BDNF signaling (Fenner, 2012). Therefore, only a

subset of TrkB-positive nerve fibers may express the full-length receptor, and BDNF may only support this subset of innervation.

Gustatory nerve fibers provide trophic signals that influence taste receptor cell maintenance. As TrkB-positive innervation was reduced after *Bdnf* removal, we investigated whether this had any impact on taste buds. We found no effect on taste bud size; however, earlier studies demonstrated a developmental impact of reducing TrkB signaling on the expression of receptors/channels required for taste transduction (Koudelka et al., 2014). We found that expression of the γ subunit of ENaC channel was reduced by *Bdnf* removal and expression of the β subunit also appeared to be reduced in the experimental group albeit not significantly due to variability. The expression of other transduction mechanisms was unaffected. Because much of a rodent's ability to transduce salt taste requires the ENaC channel (Chandrashekar et al., 2010), and ENaC channel is more effective with all 3 subunits present (Canessa et al., 1994), salt transduction may be altered in mice with reduced *Bdnf*. Our findings differ from those of earlier studies showing that disrupted TrkB-signaling resulted in an up-regulation of umami and bitter receptors and down-regulation of sour transduction mechanisms (Koudelka et al., 2014). This difference between studies is likely due to developmental stage-dependent differences in the regulation of these transduction mechanisms by BDNF-TrkB signaling. Interestingly, sodium deprivation, which reduces salt sensitivity, also impacts BDNF expression in taste buds (Huang and Stahler, 2009), implying a regulatory relationship between BDNF and ENaC expression. It is unclear whether reduction of BDNF directly influences γ-ENaC expression or whether this is an indirect effect via taste nerve fibers. As TrkB-positive innervation is specifically lost after *Bdnf* removal, TrkB-positive nerve fibers could innervate and influence γ-ENaC expression in taste receptor cells that transduce salt taste stimuli.

We showed that BDNF maintains innervation of a subset of taste nerve fibers expressing the full-length TrkB receptor. However, precisely how BDNF-TrkB signaling maintains this innervation is unclear. Because a long time period is needed to observe the impact of *Bdnf* removal (Meng et al., 2015) on innervation, it seems unlikely that BDNF-TrkB signaling is required to maintain established connections. Instead, we propose that BDNF promotes new innervation to taste buds during taste receptor cell turn over. New BDNF-expressing taste bud cells could induce taste fiber branching and growth (Cheung et al., 2007; Danzer et al., 2002; Dijkhuizen and Ghosh, 2005; Horch and Katz, 2002; Lazo et al., 2013), recruiting innervation from full-length-expressing TrkB-positive nerve fibers. If so, this would indicate that neurons expressing the full-length TrkB receptor are a specific type or subtype of gustatory neurons. In this scenario, new taste receptor cells that normally do not express BDNF may recruit TrkB-negative nerve fibers using an alternate mechanism. We propose that BDNF-TrkB signaling is one mechanism that coordinates innervation between specific types of taste receptor cells and specific types of neurons during taste receptor cell turnover.

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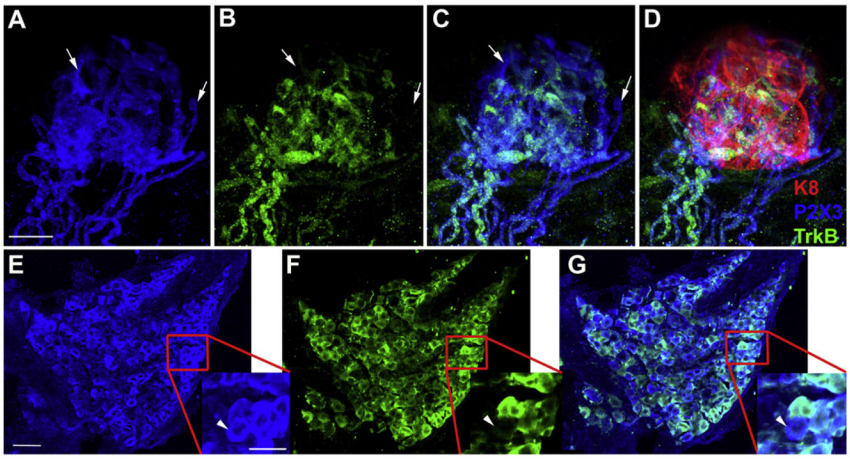

Fig. 1.

Not all P2X3-positive fibers are labeled with TrkB in fungiform taste buds. Representative fungiform taste bud labeled with anti-P2X3 (A, blue), anti-GFP (B, green), and anti-keratin 8 (C, red) in an adult *TrkB*$^{GFP/+}$ mouse. Several fibers labeled with P2X3 (A, arrows) were not labeled with GFP (B, arrows), which is seen more clearly when the blue and green images are merged (C). Keratin 8 staining demonstrates that these fibers were within the taste bud (D). Representative geniculate ganglion labeled with anti-P2X3 (E, blue) and anti-GFP (green, F) in a *TrkB*$^{GFP/+}$ mouse. The higher magnification inset for each ganglion image illustrates a neuron labeled with P2X3 but not GFP (arrowhead). Scale bar in A = 10 μm (applies to A–D); scale bar in E = 20 μm (applies to E–G); scale bar in inset = 10 μm.

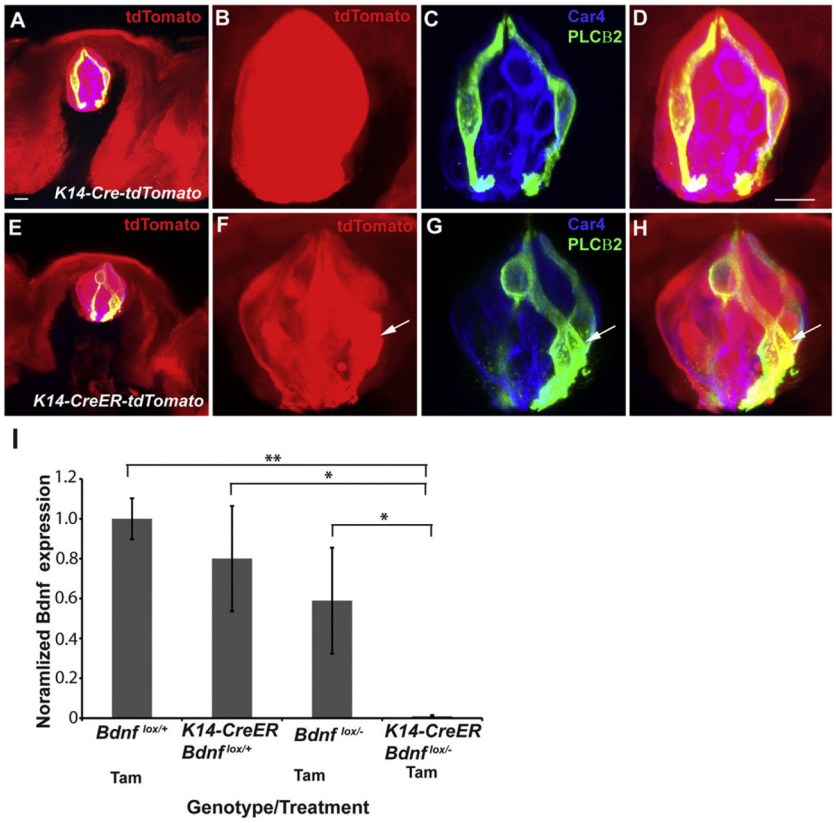

Fig. 2.

Bdnf gene expression in taste buds is reduced by inducible K14-CreER-mediated gene recombination in lingual epithelial cells after 3 weeks of tamoxifen administration. (A–D) Representative confocal images showing fungiform taste bud staining with anti-PLCβ2 (blue) and anti-Car4 (green) in an adult *K14-Cre:tdTomato* mouse. (A) TdTomato (red) was observed in the epithelium of fungiform papilla. (B–D) All taste bud cells were bright red, indicating that they were all derived from K14+ precursors. (E) Representative confocal images showing the epithelia of fungiform papillae stained with anti-K8 (blue) and anti-Car4 (green) in an adult *K14-CreER:tdTomato* mouse 10 weeks after 3 weeks of tamoxifen administration. (F–H) Most cells within the taste bud were labeled with tdTomato (red), demonstrating effective gene recombination. Many taste bud cells labeled with anti-Car4 (green) were also tdTomato-positive, indicating that they underwent gene recombination. (I) Isolated taste buds from *K14-CreER:Bdnf*$^{lox/-}$ mice expressed significantly less *Bdnf* than the three control genotypes. *Bdnf* expression was normalized to that in *Bdnf*$^{lox/+}$ mice. *$p <$ 0.05, **$p \leq 0.01$. Scale bar in A = 10 μm (applies to A and E); scale bar in D = 10 μm (applies to B–D and F–H).

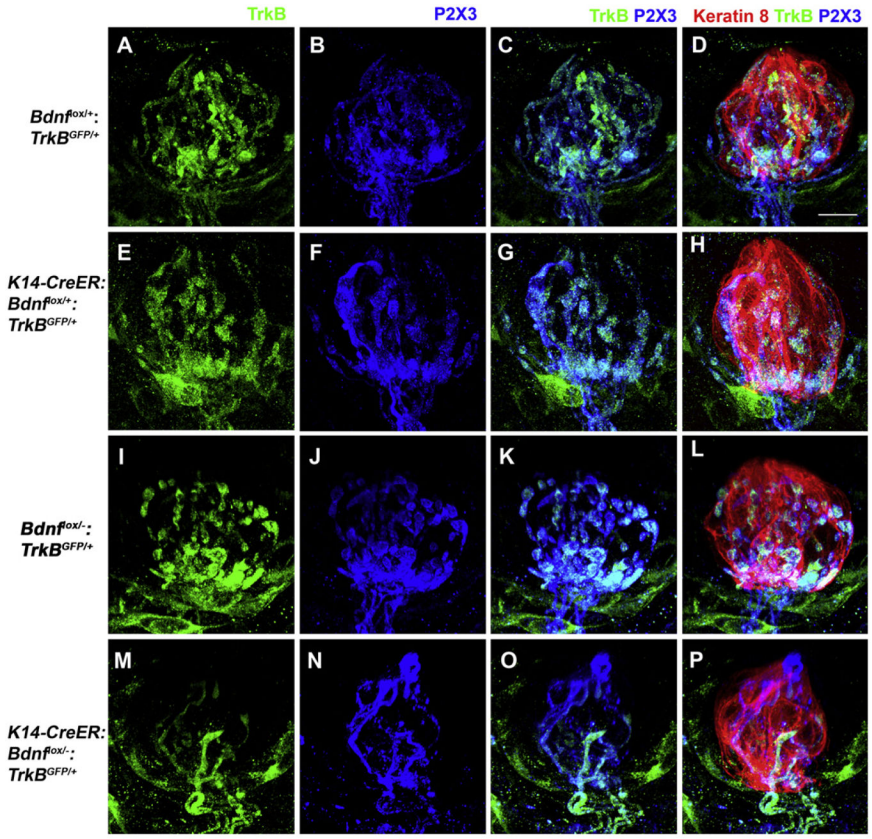

Fig. 3.

Fungiform taste buds appear to lose both P2X3-positive and TrkB-positive innervation when *Bdnf* is inducibly removed from the adult lingual epithelium. Representative confocal images showing fungiform taste buds stained with anti-GFP (green, A, E, I, and M), anti-P2X3 (blue, B, F, J, and N), and anti-Keratin 8 (red) in four different genotypes. All taste buds had robust GFP-labeled innervation, but also perigemmal cells outside the taste bud express TrkB (arrows in A, E, I, M). In $Bdnf^{lox/+}$:$TrkB^{GFP/+}$ (A–D), *K14-CreER:*$Bdnf^{lox/+}$:$TrkB^{GFP/+}$ (E–H), and $Bdnf^{lox/-}$:$TrkB^{GFP/+}$ (I–L) control mice, taste buds had similar amounts of TrkB-positive (A, E, and I) and P2X3-positive (B, F, and J) innervation 10 weeks after tamoxifen administration. However, for mice in which *Bdnf* expression was reduced (*K14-CreER:*$Bdnf^{lox/-}$:$TrkB^{GFP/+}$, M–P) taste buds had less TrkB-positive (M) and P2X3-positive (N) innervation. Interestingly, at least some TrkB-positive innervation remained in taste buds even after 10 weeks of BDNF reduction. Scale bar in D = 10 μm (applies to all panels).

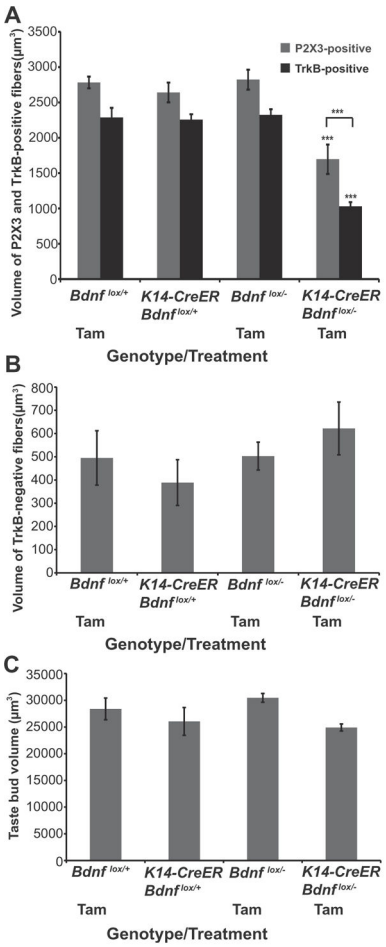

Fig. 4.

The total amount of innervation to taste buds is reduced due to a loss of TrkB-positive taste fibers. (A) The volume of P2X3-positive and TrkB-positive fibers within taste buds was reduced in adult mice with reduced *Bdnf* expression (*K14-CreER:Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$) compared with three control genotypes (P2X3-positive, *Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.005$; *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.01$; *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, $p < 0.005$; TrkB-positive, *Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.001$; *K14-CreER:Bdnf*$^{lox/+}$:*TrkB*$^{GFP/+}$, $p < 0.001$; *Bdnf*$^{lox/-}$:*TrkB*$^{GFP/+}$, $p < 0.001$). (B) The volume of TrkB-negative fibers was estimated by subtracting the volume of TrkB-positive fibers from the total volume of fibers per-taste bud. TrkB-negative innervation was not affected by *Bdnf* reduction. (C) Taste bud volume was not affected by *Bdnf* reduction. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

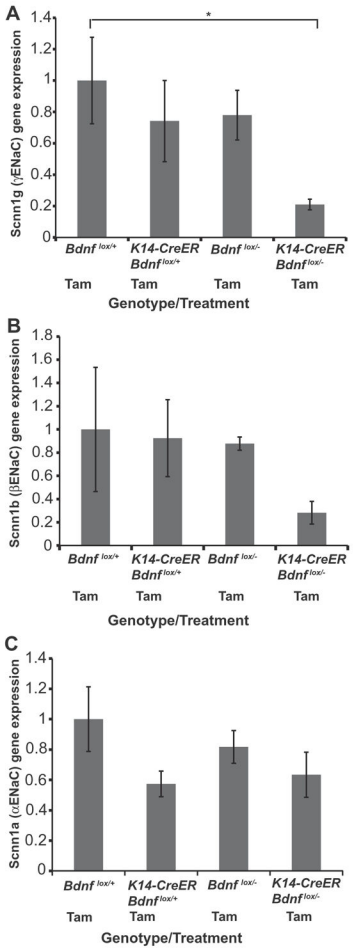

Fig. 5.

Inducibly removing *Bdnf* from the epithelium reduced expression of the γ subunit of the ENaC channel but did not affect expression of α and β subunits. Normalized expression of the γ (A), β it (B), and α subunits (C) of ENaC ($n = 4$) in fungiform taste buds. (A) RT-PCR results show reduced expression of the γ subunit of ENaC in *K14-CreER:Bdnf*$^{lox/-}$ mice compared with *Bdnf*$^{lox/+}$ mice. (B–C) RT-PCR results show similar expression of β and α subunits of ENaC expression across genotypes. *$p \leq 0.05$, **$p \leq 0.01$.

Table 1

Sequences of primer pairs and probes used for real-time RT-PCR.

Gene	Sequence 5′-3′	Fragment size (bp)
GenBank accession #		
Bdnf (NM_007540)		110
Forward primer	TGCAGGGGCATAGACAAAAGG	
Reverse primer	CTTATGAATCGCCAGCCAATTCTC	
TaqMan probe	ACTGGAACTCGCAATGCCGAACTACCCA	
T2R5 (*Tas2r105*) (NM_020501)		107
Forward primer	GGCTGGGAGTTTTAGGGAAC	
Reverse primer	AGTTGCTAAGCCGATGAGAAG	
TaqMan probe	CACTGGTAAACTGCATGGACTGGGC	
Krt8 (NM_031170)		130
Forward primer	TCTTCTGATGTCGTGTCCAAGTG	
Reverse primer	GATCCTCGGACGGGTCTCTAG	
TaqMan probe	CCACTGAAGTCCTTGCCAGCCTGAGC	
GAPDH (NM_008084)		130
Forward primer	AATGTGTCCGTCGTGGATCTG	
Reverse primer	CAACCTGGTCCTCAGTGTAGC	
TaqMan probe	CGTGCCGCCTGGAGAAACCTGCC	
β-Actin (NM_007393)		144
Forward primer	CTGGGACGACATGGAGAAGATC	
Reverse primer	GTCTCAAACATGATCTGGGTCATC	
TaqMan probe	ACCTTCTACAATGAGCTGCGTGTGGCC	
Scnn1a (NM_011324)		144
Forward primer	CCAAGGGTGTAGAGTTCTGTG	
Reverse primer	AGAGCTTGTAGTTGGTCACAC	
TaqMan probe	TTCTCCAAGTGCAGGAAGCCGTG	
Scnn1b (NM_011325)		147
Forward primer	CTCGAATATCACCCTGAGCAG	
Reverse primer	CCCATCCAGAAGCCAAACT	
TaqMan probe	CCGTACCATTGAGGAATCTCCAGCCA	
Scnn1g (NM_011326)		139
Forward primer	GGACCAAAGCCAGCAAATAAAC	
Reverse primer	ACCAAAGTTAGACAGGAGCATC	
TaqMan probe	TCATGGAGAGCCCAGCCAACAGTAT	
T1R1 (*Tas1r1*) (NM_031867)		149
Forward primer	TGATAACACTGACCACGCTG	
Reverse primer	CCTGGTACTTATCGCTGGG	
TaqMan probe	TGAGCCCTTTTCTGATGCCCCTG	
T1R2 (*Tas1r2*) (NM_031873)		128
Forward primer	AGAACCCCTTCCAAAGCATC	

Gene	Sequence 5′-3′	Fragment size (bp)
Reverse primer	AGGCTGGCAACTCTTAGAAC	
TaqMan probe	CCAACAACACGGTCCCCATATCCA	
Pkd2l1 (NM_181422)		143
Forward primer	GGTATACATAGTGTTCCTCGTGG	
Reverse primer	TGCTGATGGTTTGGAAGGAG	
TaqMan probe	TGTTTCTACACACCCCATCCGACTCTG	