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DEDICATION

This dissertation is dedicated to

my parents, Ramon Rios and Angela Pilier

and

my husband, Kenneth Brown

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ABSTRACT

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In the geniculate ganglion, taste neurons likely differentiate into subtypes during development, but very little is known about how these neurons are defined molecularly or how they differentiate. Embryonically, geniculate neuron development is regulated by the growth factor, brain derived neurotrophic factor (BDNF). Postnatally, BDNF becomes restricted to subpopulations of taste receptor cells with specific functions, primarily sour responding. I hypothesized that during development, the receptor for BDNF, tropomyosin kinase B receptor (TrkB), also becomes restricted to a neuronal subset. I used transgenic mouse models to label and quantify both geniculate neurons (primarily taste) innervating the oral cavity (Phox2b+), and those expressing TrkB (GFP) across developmental age and in conditional TrkB knockouts. I found that TrkB expression and dependence divides oral cavity projecting neurons into three subpopulations: 1) neurons that continue to express TrkB into adulthood and are TrkB-dependent during development (50%), 2) neurons dependent on TrkB during development but that downregulate TrkB expression between E15.5 and

E17.5 (41%), and 3) neurons that never express or depend on TrkB (9%). This small population of TrkB independent neurons failed to innervate any of the remaining taste buds, indicating that they may be non-taste somatosensory neurons.

It is unclear what the function of TrkB was in the 50% of neurons that continued to express TrkB in adulthood, but it could regulate neuronal function similar to its role in the adult CNS. To answer this question, I examined taste function in both chorda tympani (CT) whole nerve responses and brief-access behavioral tests after blocking TrkB-signaling. TrkB-signaling was blocked using a chemical-genetic approach in which mice with a point mutation in the TrkB (*TrkB*^{F616A}) signaling domain cause it to bind the chemical 1-NMPP1, which blocks signaling. Following administration of 1-NMPP1, CT responses were reduced for specific taste stimuli including NH_4Cl and sour stimuli (citric acid and HCl). Following conditioned taste aversion learning to 10 mM citric acid, *TrkB*^{F616A} mice treated with 1-NMPP1 had higher lick rates than vehicle treated mice to low citric acid concentrations, a finding consistent with reduced sensitivity. Functional changes were not due to changes in the morphology of TrkB fibers in the taste buds. I conclude that one adult role of BDNF-TrkB signaling in the taste bud is to directly regulate taste function.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Anatomy and organization of the peripheral taste system

Taste information from food is detected by taste receptor cells located in taste buds. These taste receptor cells convert chemical information into action potentials that propagate through taste nerves to higher order neurons in the brain. There are five different taste modalities: sweet, bitter, umami, sour, and salt. Sweet and umami tastes detect caloric value and protein content from food, respectively. Sour and bitter tastes mediate aversive signaling to prevent ingestion of harmful or poisonous substances such as spoiled food. Salty taste, on the other hand, identifies ions and minerals.

Taste buds can be found in the tongue, soft palate, larynx, and epiglottis; they are located inside specialized epithelial structures called papillae. There are three types of taste papillae: fungiform, foliate and circumvallate. The fungiform papillae comprise taste buds from the anterior 2/3 of the tongue. These papillae are innervated by the chorda tympani nerve, a branch of the facial nerve, whose cells bodies are located in the geniculate ganglion. Foliate and circumvallate papillae contain taste buds from the lateral and posterior 1/3 of the tongue,

respectively. These papillae are innervated by the glossopharyngeal nerve and their cell bodies are in the petrosal ganglion.

1.2 Taste bud cell types

Taste cells exist as a heterogenous cluster of 50-100 cells within each taste bud. These cells are classified into four types based on: their morphology, expression of transduction mechanism markers, and the neurotransmitters they release. Type I cells (dark) are electron dense, with long apical microvilli. These cells are known to have a supporting “glial-like” role as they wrap around taste cells (Bigiani, 2001; Pumplin, Yu, & Smith, 1997). Type I cells express the ectoATPase, NTPDase, important for the clearance of neurotransmitters such as ATP into ADP released by other cells (Bartel, Sullivan, Lavoie, Sevigny, & Finger, 2006; Kukulski et al., 2005). Removal of NTPDase results in accumulation of ATP and leads to desensitization of purinergic receptors in afferent nerves associated with taste cells (Vandenbeuch et al., 2013).

Type II (light) cells are electron-lucent with short apical microvilli and a large round nucleus. These taste cells also are called receptor cells because they express G protein couple receptors (GPCRs) that mediate sweet, bitter, and umami tastes. Taste stimuli (i.e. sugar) bind to the GPCR, activate phospholipase C β-2 (PLCβ2) resulting ultimately in the release of Ca^{2+}. Type II cells do not form synapses (Clapp, Yang, Stoick, Kinnamon, & Kinnamon, 2004). Instead, these taste receptor cells communicate with nerve fibers via secretion of ATP (Chaudhari, 2014; Finger et al., 2005; Kinnamon & Finger, 2013; Roper,

2006) through ion channels expressed on their surface membrane such as CALHM1/3 (Z. Ma, Saung, & Foskett, 2017; Z. Ma et al., 2018; Taruno, Matsumoto, Ma, Marambaud, & Foskett, 2013; Taruno, Vingtdeux, et al., 2013). CALHM1 is a voltage gated ion channel modulated by calcium concentration. CALHM1 facilitates the release of ATP from Type II cells in response to membrane depolarization after taste stimulation (Taruno, Vingtdeux, et al., 2013). In confirmation of this role, CALHM1$^{-/-}$ mice showed a reduction in relative responses to sweet, bitter, and umami taste responses when tested on whole nerve recordings from chorda tympani nerve (Taruno, Vingtdeux, et al., 2013). A recent study showed that CALHM1 interacts with CALHM3 to mediate ATP release from Type II cells (Z. Ma et al., 2018).

Type III (intermediate) cells are intermediate electron-lucent with a single thick apical microvillus (C. L. Yee, Yang, Bottger, Finger, & Kinnamon, 2001) .These are the only cells to form synapses with afferent fibers (Takeda & Hoshino, 1975; C. L. Yee, Jones, & Finger, 2003). Consistent with their synaptic associations with afferent nerve fibers, type III cells express proteins involved in vesicular exocytosis such as synaptosomal-associated protein (SNAP25) (DeFazio et al., 2006; R. Yang, Crowley, Rock, & Kinnamon, 2000; C. L. Yee et al., 2003) and neural cell adhesion molecules (NCAM) (G. M. Nelson & Finger, 1993). These cells release serotonin in respond to sour taste stimuli or a decrease in pH (Y. A. Huang, Maruyama, Stimac, & Roper, 2008; Y. A. Huang, Pereira, & Roper, 2011; Y. J. Huang et al., 2005; Larson et al., 2015). Type III cells respond to sour stimuli via the transient receptor potential (TRP) ion

channels, polycystic kidney disease 2-like 1 protein (PKD2L1) (Horio et al., 2011; Ishimaru et al., 2006; Kataoka et al., 2008) that also co-labeled with the carbonic anhydrase IV (Car4) (Chandrashekar et al., 2009; Lossow, Hermans-Borgmeyer, Behrens, & Meyerhof, 2017). Carbonic anhydrase IV is an enzyme that converts carbon dioxide (CO_2) into bicarbonate and H+ protons (Chandrashekar et al., 2009; Lossow et al., 2017).

Type IV (basal) cells are located at the base of the taste buds. These basal taste cells are responsible for taste bud maintenance and do not transduce any taste stimuli. As taste cells undergo renewal, progenitor cells add new taste cells into the bud by differentiating into Type I-III. These basal cells express sonic hedgehog (shh) (Castillo-Azofeifa et al., 2017; Miura & Barlow, 2010) and the neurotrophic factor, BDNF, both factors provide trophic support to taste cells and nerve fibers, respectively. Previous studies have shown that BDNF expression is downregulated in the taste buds, as result of progenitor cells (Sox2) differentiation into mature taste cells (T. Huang, Ma, & Krimm, 2015).

Salt taste is detected via epithelial ENaC channels which are amiloride-sensitive with three subunits (α, β and γ) (Chandrashekar et al., 2010; Guagliardo, West, McCluskey, & Hill, 2009; Lundy, Pittman, & Contreras, 1997; Roper, 2015). Removal of subunit α of the ENaC channel altered relative NaCl responses from chorda tympani nerve (Skyberg, Sun, & Hill, 2017; C. Sun, Hummler, & Hill, 2017). However, these studies did not specify what taste cell type lacking the ENaC channel were responsible for salt transduction. Patch-clamp studies on taste cells suggested that salt transduction might be processed

by Type I cells (Vandenbeuch, Clapp, & Kinnamon, 2008). However, there is also anatomical evidence that suggests salt could be transduced by Type III cells since ENaC-α subunit co-labeled with Car4 (sour) cells (Chandrashekar et al., 2009). In addition, BDNF-expressing cells might have a functional role in salt taste transduction since BDNF removal from the epithelium resulted in the expression of ENaC-γ subunit to be reduced (Tang, Rios-Pilier, & Krimm, 2017). It remains unclear how and what taste cells types transduce salt taste. These data support the idea that salt transduction might not be taste cell specific but instead transduced by multiple taste cells types.

Taste cells communicate with nerve fibers via neurotransmitters released in response to taste stimuli. Stimulation with sweet, bitter or umami, induce the release of ATP from Type II cells (Finger et al., 2005; Kinnamon & Finger, 2013). ATP binds to purinergic receptors P2X3/2 on nerve fibers (Bartel et al., 2006; Finger et al., 2005; Kinnamon & Finger, 2013; Vandenbeuch et al., 2015). P2X3/2 receptors are widely expressed in geniculate ganglion neurons (Ishida et al., 2009). Removal or blockage of P2X3/2 receptor ablate most taste responses while temperature, touch and menthol responses remain (Vandenbeuch et al., 2015).

1.3 Neuron types in the taste system

In the taste system, it is unclear whether or not neurons exist as distinct "types" defined by a combination of expression, function, and morphology as is the case with other sensory neurons (Le Pichon & Chesler, 2014; Zimmerman,

Bai, & Ginty, 2014). Studies have attempted to classify geniculate neuron based on their: differential development (Dvoryanchikov et al., 2017; Fei, Huang, & Krimm, 2014; Fei & Krimm, 2013; Patel & Krimm, 2010), connectivity patterns between taste cells (Larson et al., 2015; Lee, Macpherson, Parada, Zuker, & Ryba, 2017) and temporal expression of receptors (Ishida et al., 2009; Larson et al., 2015; Vandenbeuch et al., 2015). Single unit electrophysiological recordings and cluster analysis have classified these taste neurons as “specialist” or “generalist” neurons based on the sensitivity of their response to single or multiple taste stimuli presented on the tongue (Breza, Curtis, & Contreras, 2006, 2007; Contreras & Lundy, 2000; Frank, 1973; Lundy & Contreras, 1999; Wu, Dvoryanchikov, Pereira, Chaudhari, & Roper, 2015). Some of these geniculate neurons might be distinguished by characteristic and firing rates (Breza, Nikonov, & Contreras, 2010). Other functional studies suggested differential function based on expression and response to different neurotransmitters (Ishida et al., 2009; Larson et al., 2015; Vandenbeuch et al., 2015).

In chapter II, I examine whether the neurotrophin receptor, TrkB, defines geniculate neurons into subtypes based on their dependence and expression of TrkB receptor. In chapter III, using a genetic-chemical approach, I examine which taste qualities are impacted by blocking TrkB-signaling in the subset (50%) of taste neurons that express TrkB in adulthood.

1.4 Neurotrophins: role during development

Peripheral taste neurons depend on neurotrophins during development for their survival, differentiation, targeting, branching patterns, and plasticity. There are four neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT4). These neurotrophins regulate neuronal function by binding to two types of receptors: p75 and the tropomyosin related kinase (Trk). The Trk receptor family share three domains: an extracellular domain, a single transmembrane domain, and an intracellular domain with tyrosine kinase activity. P75 interacts with all four neurotrophins, NGF binds to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC (E. J. Huang & Reichardt, 2001, 2003).

Among these neurotrophins, BDNF and NT4 play critical roles in the development of the taste system (T. Huang & Krimm, 2010, 2014; Patel, Huang, & Krimm, 2010; Patel & Krimm, 2010, 2012). During embryonic development, a period of naturally cell death occurring in this ganglion, both BDNF and NT4 determine the number of geniculate neurons remaining to innervate taste buds (Patel & Krimm, 2010). In the absence of BDNF or NT4, geniculate neuron number is reduced by half, whereas removal of both BDNF and NT4 results in loss of almost all geniculate neurons (90%) (Conover et al., 1995; Liebl, Tessarollo, Palko, & Parada, 1997; X. Liu, Ernfors, Wu, & Jaenisch, 1995; Patel & Krimm, 2010, 2012). Overexpression of BDNF or NT4 augments the number of geniculate neurons (Krimm, Miller, Kitzman, Davis, & Albers, 2001; Ringstedt, Ibanez, & Nosrat, 1999). Additionally, $TrkB^{-/-}$ mice lose about 96% of geniculate neurons by E13.5 (Fei & Krimm, 2013; Fritzsch, Sarai, Barbacid, & Silos-

Santiago, 1997). However, neuronal loss in mice lacking $p75^{-/-}$ occurs slightly later in embryonic development, at E14.5 (Fei et al., 2014). After this embryonic critical period, geniculate neurons do not depend on BDNF for survival (Hoshino, Vatterott, Egwiekhor, & Rochlin, 2010; Patel & Krimm, 2010).

Consistent with the neuronal loss, BDNF regulates the development of taste buds and their innervation. $BDNF^{-/-}$ /$NT4^{-/-}$ double-knockouts develop fewer taste buds than either $BDNF^{-/-}$ or $NT4^{-/-}$ mice (Patel et al., 2010). Furthermore, $BDNF^{-/-}$ /$NT4^{-/-}$ removal during development influences taste buds in the tongue more than in the palate (Patel et al., 2010). These findings strongly suggest that neurotrophins and their receptors are crucial for the survival of developing geniculate neurons and their targets.

BDNF expression in the lingual epithelium acts as a cue for target innervation and neurite growth (L. Ma, Lopez, & Krimm, 2009; Ringstedt et al., 1999; Rochlin, O'Connor, Giger, Verhaagen, & Farbman, 2000). As nerve fibers enter the tongue, the initial innervation of taste buds is targeted by BDNF expression from the epithelium (Lopez & Krimm, 2006). In BDNF knockout mice, innervation of the tongue is achieved but afferent fibers remain under the epithelium since BDNF is not present to guide them to their taste buds targets, (Krimm et al., 2001; Lopez & Krimm, 2006; L. Ma et al., 2009). Overexpression of BDNF in the epithelium disrupts targeting and the lack of innervation results in loss of fungiform papilla and taste buds (Lopez & Krimm, 2006; L. Ma et al., 2009). Additionally, removal of the p75 receptor disrupts innervation of the mid-region of the tongue and the branching pattern during development (Fei et al.,

2014). These findings suggest that expression of BDNF taste cells is required for chorda tympani nerve fibers to properly innervate the fungiform papillae.

In other sensory ganglia, expression of the TrkB receptor defines the fate of subpopulations of neurons with distinctive function and morphology (Lallemend & Ernfors, 2012; L. Li et al., 2011). In the adult taste system, some of the geniculate neurons appear to lack the TrkB receptor (Tang et al., 2017). It is unclear how many taste neurons express the TrkB receptor and if TrkB expression is down-regulated during development. Additionally, it is unknown whether TrkB-expression could define a subpopulation of neurons in the geniculate ganglion with a different functional role in adulthood. In chapter II, I examined TrkB expression in the sensory neurons (Phox2b+) that project to the oral cavity in adulthood and when and how TrkB expression becomes restricted during development.

Regulation of neurotrophin receptors during development is used to define sensory neuron types with specific function in the dorsal root ganglion (Lallemend & Ernfors, 2012). For example, TrkB receptors become restricted to a subpopulation of neurons, the A-δ low threshold mechanoreceptors (A-δ LTMRs), that are associated with neurons that innervate hair follicles and respond to hair deflections, and rapid cooling but not warming (Abraira & Ginty, 2013; L. Li et al., 2011). In chapter II, I explored the possibility that the TrkB receptor could define neuron types in the geniculate ganglion based on TrkB expression, dependence and adult function.

1.5 Neurotrophins: role in adulthood

In the peripheral taste system, BDNF expression regulates taste bud size and innervation as new taste cells are added (T. Huang et al., 2015). Removal of BDNF in adulthood has shown that BDNF maintains taste bud innervation by TrkB fibers by adulthood (Meng, Ohman-Gault, Ma, & Krimm, 2015; Tang et al., 2017). Overexpression of BDNF in the lingual epithelium increases the volume of taste bud innervation (C. Sun, Dayal, & Hill, 2015). Since the number of geniculate neurons contributing to the chorda tympani nerve does not change in BDNF-overexpressing mice (C. Sun et al., 2015), these findings suggest that BDNF plays a role in branching of nerve fibers since it increased taste bud innervation. However, because BDNF is primarily expressed in taste bud receptor cells with synapses, previous studies have suggested a functional role of BDNF-TrkB signaling in synaptic transmission from taste cells to nerve fibers (C. L. Yee et al., 2003). However, this idea has never been experimentally tested. In chapter III, I examined the functional role of BDNF-TrkB signaling, specifically, by blocking TrkB-signaling and recording taste responses from the gustatory nerve innervating the front two-thirds of the tongue, the chorda tympani nerve.

1.6 Functional roles of BDNF-TrkB signaling

In addition to its developmental role, BDNF can directly modulate the function and structure neurons the adult CNS (Becker, Tian, Zucker, & Wang, 2016; Berninger, Schinder, & Poo, 1999; Blum, Kafitz, & Konnerth, 2002; Du &

Poo, 2004; Gokce, Runne, Kuhn, & Luthi-Carter, 2009; Kafitz, Rose, Thoenen, & Konnerth, 1999; Lohof, Ip, & Poo, 1993; H. Lu, Park, & Poo, 2014; Matsuda et al., 2009; Park, Popescu, & Poo, 2014; Rose, Blum, Kafitz, Kovalchuk, & Konnerth, 2004; Wong, Lee, Xie, Cui, & Poo, 2015; X. Zhang & Poo, 2002). In the adult CNS, BDNF influences neuronal function by regulating the morphology of dendrites to facilitate synaptic transmission (Ohira & Hayashi, 2009; J. C. Zhang et al., 2014). Additionally, BDNF enhances synaptic transmission by regulating neurotransmitter release with presynaptic and postsynaptic targets in the CNS (Amaral & Pozzo-Miller, 2012; Berninger et al., 1999; Du & Poo, 2004; Kang & Schuman, 1995; Kang, Welcher, Shelton, & Schuman, 1997; Lessmann & Brigadski, 2009; Lessmann, Gottmann, & Heumann, 1994; Y. X. Li, Zhang, Lester, Schuman, & Davidson, 1998; Lohof et al., 1993). For example, regulation of neurotransmitter release is not limited to the CNS; BDNF enhances synaptic transmission by promoting the release of acetylcholine at the presynaptic terminals in neuromuscular junctions (Lohof et al., 1993; Obis et al., 2015).

Changes in synapse morphology induced by BDNF can also potentiate synaptic transmission (Alonso, Medina, & Pozzo-Miller, 2004; Amaral & Pozzo-Miller, 2007b; Harward et al., 2016; Tyler & Pozzo-Miller, 2001; J. C. Zhang et al., 2014). Many studies have implicated a role for BDNF in depression, learning, and memory (Cazorla et al., 2011; Heldt, Stanek, Chhatwal, & Ressler, 2007; Johnson et al., 2008; Linnarsson, Bjorklund, & Ernfors, 1997; Minichiello et al., 1999; Mu, Li, Yao, & Zhou, 1999; Ren-Patterson et al., 2005; Shin, Kim, & Kim, 2011; C. Yang, Shirayama, Zhang, Ren, & Hashimoto, 2015; J. C. Zhang et al.,

2014; J. C. Zhang et al., 2015). Upregulation of BDNF improves spatial learning and memory and other cognitive deficits associated with anxiety and depression (Cazorla et al., 2011; Heldt et al., 2007; Johnson et al., 2008; Linnarsson et al., 1997; J. C. Zhang et al., 2014; J. C. Zhang et al., 2015). In addition, induction of BDNF by Neuropep-1 improves spatial learning and memory in rats (Shin et al., 2011). Studies with the selective TrkB receptor antagonist ANA-12 suggested that BDNF exerts its anti-depressant and anxiolytic effects by modifying dendritic spines (Cazorla et al., 2011; C. Yang et al., 2015; J. C. Zhang et al., 2014).

BDNF-TrkB signaling also can have rapid effects on neuron function by regulating the activation of channels that play a role in excitability and resting potential such as Nav1.9 (Blum et al., 2002; Kafitz et al., 1999), Nav1.2 (Ahn, Beacham, Westenbroek, Scheuer, & Catterall, 2007), TRPC3 (Amaral & Pozzo-Miller, 2007a; H. S. Li, Xu, & Montell, 1999) and suppression of Kiv1.3 channels (Mast & Fadool, 2012; Tucker & Fadool, 2002). Application of BDNF induced depolarization of central neurons within milliseconds and increases the firing rate of neurons in a dose-dependent manner (Kafitz et al., 1999). BDNF-induced neuronal depolarization requires activation of the full-length TrkB receptors and the opening of Nav1.9 channels (Blum et al., 2002; Kafitz et al., 1999). Additionally, these functional effects are reversibly blocked by K252a, a tyrosine kinase inhibitor (Kafitz et al., 1999) and saxitoxin (STX), a selective sodium channel blocker (Blum et al., 2002). Another channel involved in BDNF-induced neuronal excitability is the voltage-dependent potassium channel Kiv1.3. In the olfactory bulb, activation of BDNF-TrkB signaling suppresses Kiv1.3 activity

within 15mins due to phosphorylation (Tucker & Fadool, 2002). Moreover, phosphorylation of TrkB receptors recruits adaptor proteins that activates the PLCγ pathway to depolarize the membrane via TRPC3 receptors (H. S. Li et al., 1999). The PLCγ inhibitor, SKF-96365, blocked the changes in current induced by BDNF application (Amaral & Pozzo-Miller, 2007b; H. S. Li et al., 1999).

Studies in sensory neurons, suggest that BDNF might play a role in nociception (Buldyrev et al., 2006; Fabbretti & Nistri, 2012; Pezet, Malcangio, & McMahon, 2002; Simonetti, Giniatullin, & Fabbretti, 2008; Wang, Ratnam, Zou, England, & Basbaum, 2009), specially via purinergic receptors and calcitonin gene related peptide (CGRP) (Simonetti et al., 2008). CGRP upregulates gene expression of purinergic receptors, P2X3 (Simonetti et al., 2008) and the release of BDNF from trigeminal neurons (Buldyrev et al., 2006). Moreover, blocking TrkB-signaling reduces hyperalgesia induced by capsaicin or formalin (Wang et al., 2009). These studies suggest that BDNF via activation of TrkB can modulate nociception by regulating the release of neurotransmitters and function of channels.

CHAPTER II

TRKB EXPRESSION AND DEPENDENCE DIVIDES GUSTATORY NEURONS INTO THREE SUBPOPULATIONS

2.1. Introduction

The geniculate ganglion primarily contains two neuron subpopulations: 1) neurons that carry mostly taste, but some somatosensory information from the anterior two-thirds of the tongue and the palate to the brain (oral sensory), and 2) those that innervate the outer ear through the auricular nerve (auricular neurons). Development of most of these neurons is regulated by the neurotrophin brain-derived neurotrophic factor (BDNF). BDNF binds with high affinity to the tropomyosin kinase B receptor (TrkB) (Barbacid, 1995; E. J. Huang & Reichardt, 2003) and critically regulates the survival of taste neurons (Patel et al., 2010; Patel & Krimm, 2010). Early in development, BDNF expression in taste buds acts as a cue for TrkB+ fibers to innervate taste organs (Hoshino et al., 2010; Krimm et al., 2001; Lopez & Krimm, 2006; L. Ma et al., 2009; C. A. Nosrat, Ebendal, & Olson, 1996; C. A. Nosrat & Olson, 1995; Ringstedt et al., 1999; Rochlin et al., 2000). Both this target innervation process and neuron survival occur during a critical developmental period (Hoshino et al., 2010; L. Ma et al., 2009). After this critical period, BDNF is downregulated in the taste bud, but continues to be

expressed in a subpopulation of taste receptor cells (T. Huang et al., 2015; C. L. Yee et al., 2003). This expression change suggests that BDNF may play a different role/s in later developmental stages and adulthood.

Most geniculate ganglion neurons express the BDNF receptor TrkB (Farbman et al., 2004; Fei & Krimm, 2013; Yamout, Spec, Cosmano, Kashyap, & Rochlin, 2005) early in development and depend on TrkB signaling for their survival (Fei & Krimm, 2013; Fritzsch et al., 1997; C. A. Nosrat, 1998). However, in TrkB knockout animals, many innervated taste buds remain at birth despite profound geniculate neuron loss by E13.5 (Fei & Krimm, 2013; Fritzsch et al., 1997). One possible explanation is that gustatory neurons lacking the TrkB receptor could migrate into the geniculate ganglion after E13.5. Consistently, in chick embryos, geniculate neurons continue to delaminate from the epibranchial placodes and migrate into the geniculate ganglion throughout embryonic development (Blentic, Chambers, Skinner, Begbie, & Graham, 2011). How many gustatory neurons remain after E13.5 in TrkB knockouts is unclear, particularly because earlier studies lacked markers for taste neurons and did not quantify geniculate neurons after E13.5 (Fei & Krimm, 2013; Fritzsch et al., 1997). Therefore, a subset of TrkB-independent taste neurons of unknown size likely exists.

By adulthood, only a subset of taste neurons appears to be regulated by BDNF, as adult BDNF removal only reduces taste bud innervation by 40% (Meng et al., 2015; Tang et al., 2017). Therefore, in adulthood, many gustatory neurons likely lack the TrkB receptor. Yet, it remains unknown if, when, or how, during

development or adulthood, TrkB expression decreases in the neurons that innervate the oral cavity. Here, my primary goals were to determine 1) how many neurons express TrkB in adulthood, and 2) when and how TrkB expression decreases during development.

Previous studies examining TrkB expression in adulthood lacked markers for distinguishing the roughly 50% of geniculate ganglion neurons that are oral sensory from the auricular neurons innervated the outer ear. The transcription factor Phox2b was recently established as a marker that distinguishes geniculate ganglion neurons innervating the oral cavity (Phox2b+) from those innervating the ear (Phox2b-) (Dvoryanchikov et al., 2017; Ohman-Gault, Huang, & Krimm, 2017). Using Phox2b as a marker for geniculate taste neurons, I determined that developmental reduction of TrkB-expression and differential dependence on TrkB for survival divide oral sensory neurons of the geniculate into three subsets. These subpopulations were: 1) neurons that depend on TrkB expression during development and continue to express TrkB receptors in adulthood (50%), 2) neurons that depend on TrkB during development but downregulate it between E15.5 and birth (41%), and 3) neurons that never express or depend on TrkB and do not innervate taste buds (9%). Therefore, adult roles of TrkB-signaling are likely restricted to a subset of oral cavity projecting neurons.

2.2. Materials and methods

2.2.1. Animals

All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature, with standard alternating 12-h periods of light/dark and free access to water and food. *Animals were cared for and used in accordance with guidelines of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and NIH Guide for the Care and Use of Laboratory Animals.*

To visualize TrkB expression in Phox2b-expressing neurons, Phox2b-Cre (Tg[Phox2b-cre]NP91Gsat/Mmucd, Stock No. 034613-UCD(Ohman-Gault et al., 2017)) with tdTomato (Ai14, Jax Stock No. 007914) mice were bred with $TrkB^{GFP/+}$ (B6.129S6[Cg]-*Ntrk2*$^{tm2.1Ddg}$/J, Jax Stock No. 023046) mice (L. Li et al., 2011; Madisen et al., 2010). In Phox2b-Cre mice, gene recombination occurs in any neuron that has ever expressed Phox2b. In the geniculate ganglion, this is specific to oral sensory and excludes the somatosensory neurons that innervate the outer ear via the auricular nerve (Ohman-Gault et al., 2017). These oral sensory neurons are mostly gustatory, but also include somatosensory fibers (Donnelly, Shah, Mistretta, Bradley, & Pierchala, 2018; Dvoryanchikov et al., 2017; Kumari et al., 2015; Kumari et al., 2017). However, most of the somatosensory neurons innervating the tongue arise from the trigeminal ganglion.

Embryo heads from mice aged E13.5 (n = 2), E15.5 (n = 3), E17.5 (n = 4), and P0 (n = 3) were collected for immunohistochemistry. The day that a vaginal plug was observed was designated E0.5. Geniculate ganglia from adult mice (P60) were also collected for whole mount and serial sections. To conditionally

remove the TrkB receptor from oral cavity neurons, I bred Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice with a mouse line in which the coding region of the *TrkB* gene was surrounded by loxP sites (*Ntrk2*^{tm1Ddg}/J mouse, Jax Stock No. 022363) (Chen et al., 2005). These conditional TrkB knockout mice do not live to adulthood and instead die at different postnatal ages (Fei & Krimm, 2013; Ozek, Zimmer, De Jonghe, Kalb, & Bence, 2015), so I collected geniculate ganglia and tongues from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ and Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ at P20. These geniculate ganglia were processed to determine how many oral sensory neurons depended on TrkB signaling during development.

2.2.2. Immunohistochemistry

Embryos aged E13.5 were fixed by immersion in 4% paraformaldehyde (PFA). Embryos aged E15.5 and older, young (P20) mice, and adult (P60) mice were all sacrificed with an overdose (1 ml, i.p.) of 2.5% tribromoethanol (Avertin) and then were trans-cardially perfused with 4% PFA. Geniculate ganglia were dissected under a microscope and then embryo and newborn heads or geniculate ganglia from adult mice were post-fixed in 4% PFA overnight at 4 °C. Tissue was then transferred to 30% sucrose/phosphate-buffered saline (PBS) for cryoprotection at 4 °C overnight. The following day, tissue was frozen on dry ice in OCT (Sakura Tek) embedding medium and stored at -80 °C until processing for immunohistochemistry.

To visualize oral cavity neurons in adult mice, entire geniculate ganglia were rinsed in 0.1 M PB four times and blocked overnight with 3% donkey serum in 0.1 M PBS containing 0.5% Triton X-100 (PBST). Ganglia were then incubated with the following primary antibodies for 5 d at 4 °C: goat anti-GFP (1:400; Novus, AB Registry ID: AB_10128178, Cat No. NB100-1700, Littleton, CO) and rabbit polyclonal anti-dsRed (1:500, Clontech Laboratories, Inc., Cat No. 632496, Mountain View, CA). After incubation in primary antibodies and four rinses with 0.1 M phosphate buffer (PB), tissues were incubated for 2 d in the following secondary antibodies: goat anti-GFP Alexa Fluor 488 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-rabbit Alexa Fluor 555 (1:500; Jackson ImmunoResearch Laboratories). The tissue was then washed four times in 0.1 M PB, mounted onto slides, and cover-slipped using aqueous mounting medium (Fluoromount-G, SouthernBiotech, Birmingham, AL).

Alternatively, serial transverse sections (20 µm) were cut using a cryostat. Sections were left to air-dry on a slide warmer overnight. The next day, sections were post-fixed in 4% PFA for 15 min at 4 °C. After four rinses with PBST, sections were blocked for 1 h at room temperature with 5% normal goat serum in PBST. Then, the tissue was incubated overnight at 4 °C with the following primary antibodies: chicken anti-GFP (1:1,000; Thermo Fisher, Cat No. A10262, Carlsbad, CA) and rabbit anti-P2X3 (1:500; Millipore, AB Registry ID: AB_11212062, Cat No. AB58950, Billerica, MA). After incubation in primary antibodies and four rinses in PBST, sections were incubated for 1 h at room

temperature in the following secondary antibodies: goat anti-chicken Alexa Fluor 488 (1:500; Jackson ImmunoResearch Laboratories) and goat 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).

To visualize fungiform papillae and innervated taste buds, anterior tongue-halves from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ and Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice were collected at P20 using the same procedures as described for geniculate ganglia. To examine taste bud innervation and Car4-positive taste cells, thick sagittal sections (70 µm) of one-half tongue from each animal was cut using a cryostat and rinsed in 0.1 M PB four times for 15 min. After blocking with 3% normal donkey serum in 0.1 M PB containing 0.5% Triton X-100 overnight at 4° C, tissues were incubated for 5 d in the following primary antibodies: rat anti-cytokeratin-8 (K8, 1:50, Cat No. Troma-1-s, Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal anti-dsRed (1:500, Clontech Laboratories, Inc., Cat No. 632496), and either goat anti-Car4 (1:500, Cat No. AF2414, R&D Systems, Minneapolis, MN). Floating sections were rinsed in 0.1M PB four times for 15 min and incubated for 2 d in the following secondary antibodies (Jackson ImmunoResearch Laboratories): donkey anti-rat Alexa Fluor 488, donkey anti-rabbit Cy3, and donkey anti-goat Alexa 647. To examine taste bud innervation and PLCβ2-positive taste receptor cells, the other half of the tongue for each animal was incubated for 5 d in rabbit anti-PLCβ2 (1:500, Cat No. SC-206, Santa Cruz Biotechnology, Dallas, TX). Floating sections were

rinsed in 0.1M PB four times for 15 min and incubated for 2 d in donkey anti-rabbit Alexa Fluor 647. Then, sections were rinsed in 0.1 M PB four times for 15 min and blocked for 2 d with donkey anti-rabbit (1:100, Cat No. 007-003-007, Jackson ImmunoResearch, Laboratories). After four rinses in 0.1M PB for 15 min, sections were incubated for 5 d in the following primary antibodies: rat anti-K8 (1:50, Cat No. Troma-1-s, Developmental Studies Hybridoma Bank, Iowa City, IA), and rabbit polyclonal anti-dsRed (1:500, Clontech Laboratories, Inc., Cat No. 632496). Floating sections were rinsed in 0.1M PB four times for 15 min and incubated for 2 d in the following secondary antibodies (Jackson ImmunoResearch Laboratories): donkey anti-rat Alexa 488, donkey anti-goat Alexa Fluor 488, and donkey anti-rabbit Cy3. To quantify fungiform papillae, all tissues were then rinsed in 0.1 M PB and stained with DAPI (2 µL in 50 mL of double distilled H20, Life Technologies, Foster City, CA) for 45 min. After four rinses in 0.1 M PB, tissues were cover-slipped using aqueous mounting medium (Fluoromount-G).

2.2.3. Neuron quantification of whole mounts at P20 and in adulthood

Images of whole ganglia were captured using a 40× oil immersion lens (FV1200, Olympus) with a step size of 0.47 µm. Confocal images were obtained by stitching multiple fields to create a high-resolution image of the entire ganglia in a single Z-stack. Each labeled channel was collected individually using single-wavelength excitation. Images of each optical section through the whole ganglia were then analyzed using Neurolucida 360 (MBF Bioscience, Williston, VT).

Brightness and contrast were adjusted for background standardization in all images. Each labeled neuron was followed through the optical sections so that each neuron was only counted once. The absolute number of single- (anti-dsRed) and double-labeled (anti-dsRed and anti-GFP) neurons were counted by examining each cell through the Z-stack.

2.2.4. Neuron quantification of sections during development

Serial sections of embedded embryo and newborn heads and adult geniculate ganglia were captured using a 40× oil immersion lens (FV1200, Olympus). Confocal images were collected using single-wavelength excitation taken with a step size of 1.0 µm. Alternating images from serial sections containing ganglia were then analyzed using Neurolucida 360 (MBF Bioscience). The number of single- (anti-dsRed) and double-labeled (anti-dsRed and anti-GFP) neurons were counted in alternating sections by following each cell through the Z-stack so that each neuron was only counted once. The percentage of oral cavity neurons (tdTomato+) expressing TrkB (GFP+) was calculated for each section and averaged for each animal per age group and then compared to tdTomato single-positive neurons. To quantify TrkB expression in non-taste neurons, the percentage of neurons that expressed both TrkB (GFP) and P2X3 but not Phox2b was examined across ages.

2.2.5. Quantification of fungiform papillae and taste bud number after conditional TrkB removal from oral sensory neurons

Images of taste buds were captured from thick sections of tongue-halves using a 60× oil immersion lens with a step size of 1.0 µm. Images were collected using single-wavelength excitation and projected along the Z-axis. To determine how TrkB removal affected taste bud number, I counted taste buds in the fungiform papillae using DAPI staining with a fluorescence microscope and a 20× objective lens. I examined all sections for one-half of a mouse tongue. Anti-K8 labeling indicated the presence of a taste bud in each fungiform papilla. I examined all the taste buds labeled with anti-K8 on each half tongue and quantified the number of taste buds containing Car4+ or PLCβ2+ labeled cells. The percentage of taste buds containing Car4+ and PLCβ2+ taste receptor cells was calculated by dividing the number of taste buds containing labeled cells by the total number of taste buds for each one-half of tongue (anti-K8, n = 5/genotype).

2.2.6. Data Analysis

A student's t-test was used to compare the number of double-labeled vs. single-labeled neurons in Phox2b-Cre::tdTomato::*TrkB*$^{GFP/lox}$ mice in adulthood (n= 3). A Chi-squared (X^2) test was used to analyze the percentage of Phox2b neurons that express TrkB in geniculate ganglion sections in E15.5 (n = 3), compared to E17.5 (n = 4), P0 (n = 3) and adult (n= 3). Because this is 3 statistical tests, type one error was controlled using a Bonferroni correction, and so a p-value of $p < 0.0167$ was established before analysis. A Chi-squared (χ^2) test was also used to analyze TrkB expression in Phox2b-negative neurons

across different ages (E13.5 (n = 2), P0 (n = 3), and adult (n= 3)) and Bonferroni correction was used for a p-value of $p < 0.025$. A students t-test was used to compare the number of remaining neurons at P20 in conditional TrkB knockouts (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/lox}$, n=7) compared with controls (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$, controls, n = 5). A student's t-test was used to compare the number of taste buds between controls (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$, n=5) and conditional knockouts (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/lox}$ n=5). Chi-squared (χ^2) test was used to compare the percentage of taste buds with PLCβ2+ and Car4+ cells between (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/lox}$, conditional knockouts, n=4) and controls (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$, controls, n=4). Except where otherwise specified, a significance for all tests was established as $p < 0.05$ before analysis; however, actual p-values are reported.

2.3. Results

2.3.1. Only half of the oral sensory neurons in the geniculate ganglion express TrkB in adulthood

Early in development, approximately 90% of geniculate neurons express TrkB and depend on TrkB for their survival (Fei & Krimm, 2013; Fritzsch et al., 1997). I hypothesized that following this critical period for neuron survival, only a subset of the neurons projecting to the anterior two-thirds of the tongue would continue to express TrkB in adulthood. This would be consistent with the finding that not all adult gustatory nerve fibers appear to express TrkB in adulthood (Tang et al., 2017).

Recently, Phox2b expression has been used to distinguish oral sensory neurons (Phox2b+) from those innervating the outer ear (auricular neurons; Phox2b-)(Dvoryanchikov et al., 2017; Ohman-Gault et al., 2017). Trigeminal neurons innervating the tongue are also Phox2b-. Thus, in the geniculate ganglion, Phox2b expression is fairly specific to taste neurons, plus a few general oral sensory neurons. To visualize TrkB expression in oral sensory neurons, I crossed Phox2b-Cre::tdTomato mice with *TrkB*$^{GFP/+}$ mice to visualize TrkB receptor expression (GFP+: green) in oral sensory neurons (dsRed+, red; Figure 1A). I quantified neurons from Z-stacks of adult (P60) whole mount geniculate ganglia. In adulthood, not all geniculate ganglion neurons expressed the TrkB receptor (Figure 1A, B). Specifically, while GFP labeling was distributed throughout the ganglion, tdTomato+ neurons were located more medially and closer to the greater superficial petrosal nerve (Figure 1, A). This suggests that oral cavity-projecting neurons are primarily localized to one part of the ganglion.

I then quantified the numbers of single-labeled (TrkB-, tdTomato only) and double-labeled (TrkB+, red and green) oral cavity-projecting neurons in whole mount P60 geniculate ganglia (Figure 1B). An average of 354 ± 33 neurons were Phox2b+. Of those, 185 ± 21 neurons co-labeled with GFP, while 169 ± 0 neurons expressed tdTomato alone ($p < 0.01$; Figure 1B). Thus, roughly 52% of oral cavity-projecting neurons express TrkB in adulthood.

2.3.2. TrkB expression declines between E15.5 and E17.5.

Although approximately half of oral cavity-projecting neurons express TrkB in adulthood (Figure 1), approximately 90% geniculate neurons express TrkB between E11.5 and E13.5 (Farbman et al., 2004; Fei & Krimm, 2013; T. Huang & Krimm, 2010; Yamout et al., 2005). I next sought to determine at what period during development TrkB expression decreases. Using Phox2b-tdTomato to identify oral cavity-projecting neurons, I quantified the number of tdTomato+ neurons expressing TrkB-GFP at ages E13.5, E15.5, E17.5, at birth (P0), and adulthood (P60) from serial transverse sections using immunohistochemistry. Consistent with previous findings (Fei & Krimm, 2013; Yamout et al., 2005), I observed *TrkB*^{GFP} labeling throughout most of the geniculate ganglion at E13.5. *TrkB*^{GFP} labeling was also observed outside of the geniculate ganglion (Top panel, Figure 2A-E). Across all ages examined, Phox2b-tdTomato labeling (red) was restricted to the medial portion of the geniculate ganglion (Middle panel, Figure 2 A-E). At E13.5 and E15.5, *TrkB*^{GFP} labeling appeared uniformly distributed throughout the geniculate ganglion. After E17.5, TrkB labeling appeared to be more strongly expressed tdTomato- (non-taste) neurons, and this pattern persisted through adulthood (bottom panel, Figure 2A-E).

Understanding the temporal dynamics of TrkB expression might provide insight into the mechanism underlying decreased TrkB expression in adulthood. To determine when during development TrkB expression begins to decrease in oral sensory neurons, the percentage of Phox2b+ neurons co-labeled with GFP was quantified E13.5-P60 (Figure 2F). At E13.5, most (95%) tdTomato+ neurons expressed TrkB. The remaining 5% of tdTomato+ neurons likely consisted of the

small subpopulation of TrkB-independent geniculate ganglion taste neurons (Fei & Krimm, 2013). TrkB expression in oral cavity-projecting neurons did not differ between ages E13.5 and E15.5 (91%; $p = 0.151$). However, by E17.7, TrkB expression was significantly reduced to only 63% ($p < 0.001$) and remained reduced at birth (57%; $p < 0.001$). By adulthood, only 50% of tdTomato+ neurons expressed GFP. These data replicate our findings regarding TrkB expression in whole mount ganglia (Figure 1). Thus, TrkB expression is significantly reduced to approximately 50% of oral cavity-projecting neurons between E15.5 and E17.5 and remains reduced into adulthood.

2.3.3. Decreased TrkB expression is specific to Phox2b+ oral sensory neurons

Since TrkB expression is reduced in tdTomato+ neurons, I next wanted to determine if this decrease was specific to oral sensory neurons, or if it also occurred in the auricular neurons of the geniculate ganglion. Previous studies have shown that these afferents express TrkB receptors as early as E11 (Yamout et al., 2005). To determine if TrkB expression was also reduced in these auricular neurons during development, I examined TrkB expression in tdTomato- neurons. I used P2X3 (Figure 3: blue) as a general marker of geniculate ganglion neurons (Fei & Krimm, 2013; Ishida et al., 2009; Vandenbeuch et al., 2015). Contrary to previous reports (Ishida et al., 2009), P2X3 did not label all tdTomato- neurons (Figure 3). P2X3 labeling was observed in many sensory neurons within the geniculate ganglion, including some Phox2b- cells. *TrkB*^{GFP}

labeling was brighter in some regions of the geniculate ganglion, especially near Phox2b- neurons. This pattern continued into birth and adulthood. I counted the number of P2X3+/tdTomato- (non-taste neurons) with and without GFP (TrkB). Early in development (E13.5), most (92.9%) P2X3+ neurons also express *TrkB*^{GFP}. Importantly, the percentage of P2X3+/tdTomato- neurons expressing TrkB did not significantly differ at birth (86.4%; $p > 0.05$) or adulthood (94.7%; $p > 0.05$). Thus, in somatosensory (non-taste) geniculate ganglion neurons, TrkB receptor expression remains consistent across development. I conclude that decreased TrkB expression during development is specific to a subset of neurons that carry oral sensory (primarily taste) information from the tongue and soft palate to the brain. TrkB reduction is likely part of the differentiation program for this specific subset of taste neurons.

2.3.4. A small population of Phox2b+ sensory neurons is TrkB-independent during development

When TrkB expression is eliminated during development, most geniculate ganglion neurons (94%) are lost by E13.5. However, 33% of the taste buds remain and most of these are innervated at birth (Fei & Krimm, 2013), which seemed a surprising amount of innervation considering the large total geniculate neuron loss. Because neurons continue to migrate into the geniculate ganglion over a long period of embryonic development (Blentic et al., 2011), I speculated that at least some of the decreased TrkB+ expression observed at E17.5 resulted from the migration of TrkB- neurons into the geniculate ganglion after E15.5. If

this were the case, these TrkB+ tdTomato+ neurons would be present in the geniculate ganglion at later stages of development when TrkB is conditionally removed. Alternatively, TrkB expression may be downregulated after the critical developmental period for either geniculate neuron targeting or survival (Hoshino et al., 2010; L. Ma et al., 2009; Meng et al., 2015; Runge, Hoshino, Biehl, Ton, & Rochlin, 2012). In this case, most geniculate neurons would be lost in conditional TrkB knockouts. To evaluate these possibilities, I quantified the number of tdTomato+ neurons remaining in the geniculate ganglion at P20 in conditional TrkB knockout mice (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$) compared to controls (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$).

As shown in adulthood (Figure 1), TrkB expression was not homogenous across the geniculate ganglion at P20 (Figure 4A, green). tdTomato expression was restricted to a subset of neurons, confirming that not all geniculate ganglion neurons express Phox2b (Figure 4A, red). The geniculate ganglia from Phox2b-Cre::tdTomato:: *TrkB*$^{GFP/loxP}$ mice were smaller and contained fewer tdTomato+ neurons relative to controls. Interestingly, an average of 28 ± 5.3 (n=7) tdTomato+ neurons remained in the geniculate ganglion after TrkB removal compared to 349.8 ± 44.6 (n=5) in control mice (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$; Figure 4C; ***$p < 0.001$). The remaining 8% of tdTomato+ neurons represent a small subset of TrkB-independent neurons. This proportion is equivalent to the percentage of tdTomato+/TrkB- neurons in the geniculate ganglion at E13.5 (5%). Therefore, new TrkB-independent taste neurons do not migrate into the geniculate ganglion after E13.5. These results

support the conclusion that 92% of taste neurons express and depend on TrkB during development (E13.5 and earlier), and that TrkB is downregulated in a subset of these neurons between E15.5 and E17.5.

2.3.5. Remaining neurons innervate fungiform papillae but not taste buds, resulting in a substantial taste bud loss by P20.

A small group of Phox2b+ geniculate neurons do not depend on the TrkB receptor during development. These oral sensory neurons are primarily gustatory, but also include a small population of somatosensory neurons (Dvoryanchikov et al., 2017; Yokota & Bradley, 2016). Therefore, whether the remaining neurons or supported/innervated taste buds was unclear. To examine the location of innervation for these remaining oral sensory neurons, I examined the entire tongue for dsRed positive nerve fibers in control (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$; Figure 5A) and conditional TrkB knockout (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$; Figure 5B, C) mice. Conditional TrkB removal reduced taste bud size and completely eliminated tdTomato innervation within taste buds (Figure 5B). I found only a few tdTomato+ fibers innervating the lingual epithelium. These fibers innervated fungiform papillae, but these papillae lacked taste buds (Figure 5C). The few neurons remaining following TrkB removal might belong to a somatosensory neuron population, since they innervated fungiform epithelium and not taste buds.

TrkB is required for the survival of 92% oral sensory neurons in the geniculate ganglion; gustatory neurons are required to maintain taste buds (Fei

et al., 2014; Ito & Nosrat, 2009; I. V. Nosrat, Agerman, Marinescu, Ernfors, & Nosrat, 2004; Oakley, Lawton, Riddle, & Wu, 1993). So, next I quantified the number of taste buds that developed in the absence of TrkB fibers. Consistent with previous findings, TrkB removal significantly reduced the number of taste buds (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$: 63.8 ± 4.9 vs. Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$: 8.9 ± 1.5, **p < 0.01, Figure 5D). TrkB removal from Phox2b+ neurons reduced taste buds by 86%, suggesting that TrkB-dependent neurons support most taste buds during development.

Since the remaining taste buds completely lacked innervation, I also wanted to determine if nerve fibers preferentially supported specific subtypes of taste receptor cells. Taste buds from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ (Figure 5A, E-G) and Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ (Figure 5B, H-J) mice were labeled for PLCβ2, a marker for Type II taste receptor cells that transduce bitter, sweet, umami (DeFazio et al., 2006), and for Car4, a marker of Type III cells that transduce sour (Chandrashekar, Hoon, Ryba, & Zuker, 2006). I quantified the percentages of taste buds with PLCβ2+ and Car4+ cells for both genotypes. All taste buds from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice contained both Car4+ and PLCβ2+ taste receptor cells (Figure 5F and G). In contrast, taste buds from conditional knockouts typically lacked Car4+ cells (Figure 5I and J), while PLCβ2+ cells were present in 100% of taste buds examined from both genotypes (Figure 5K). Conditional TrkB knockout significantly reduced the percentage of Car4+ cells in the remaining taste buds to ~21.3 ± 2.5% compared to controls (Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$: 85.3 ± 1.8%, **p < 0.01, Figure 5K). These

data suggest that the loss of innervation caused by TrkB removal markedly reduces the number of taste buds and Type III taste receptor cells by P20.

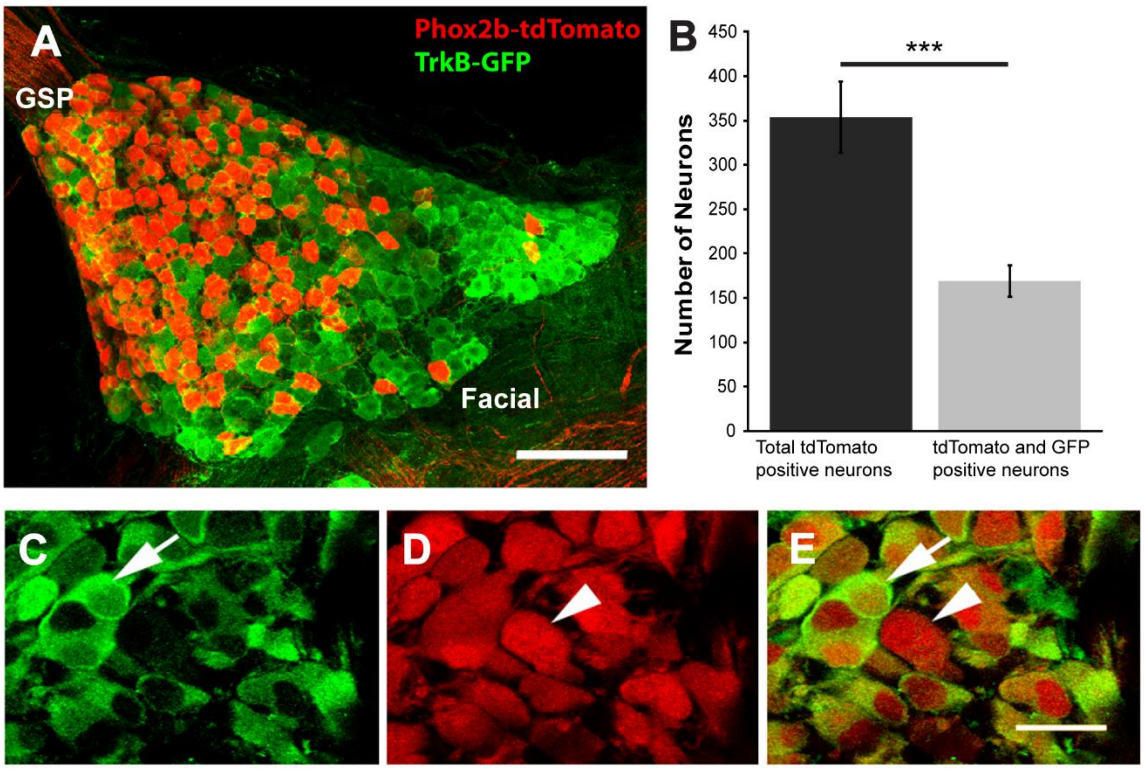

Figure 2.1. **Half of adult taste neurons express the TrkB receptor.** A) Representative image of a whole mount adult geniculate ganglion from a Phox2b-Cre::tdTomato::TrkB$^{GFP/+}$ mouse labeled for both tdTomato (oral cavity-projecting, red) and GFP (TrkB, green). B) Quantification of the total number of neurons (n=3) projecting to the oral cavity (red only) and oral cavity-projecting neurons that express TrkB^{GFP} (double-labeled). C-E) Magnified images illustrate the criteria used to identify and count single-labeled (arrow) and double-labeled (arrowhead) oral cavity-projecting neurons. Scale bar = 50 µm (A) and 10 µm (C-E). ***p <0.001.

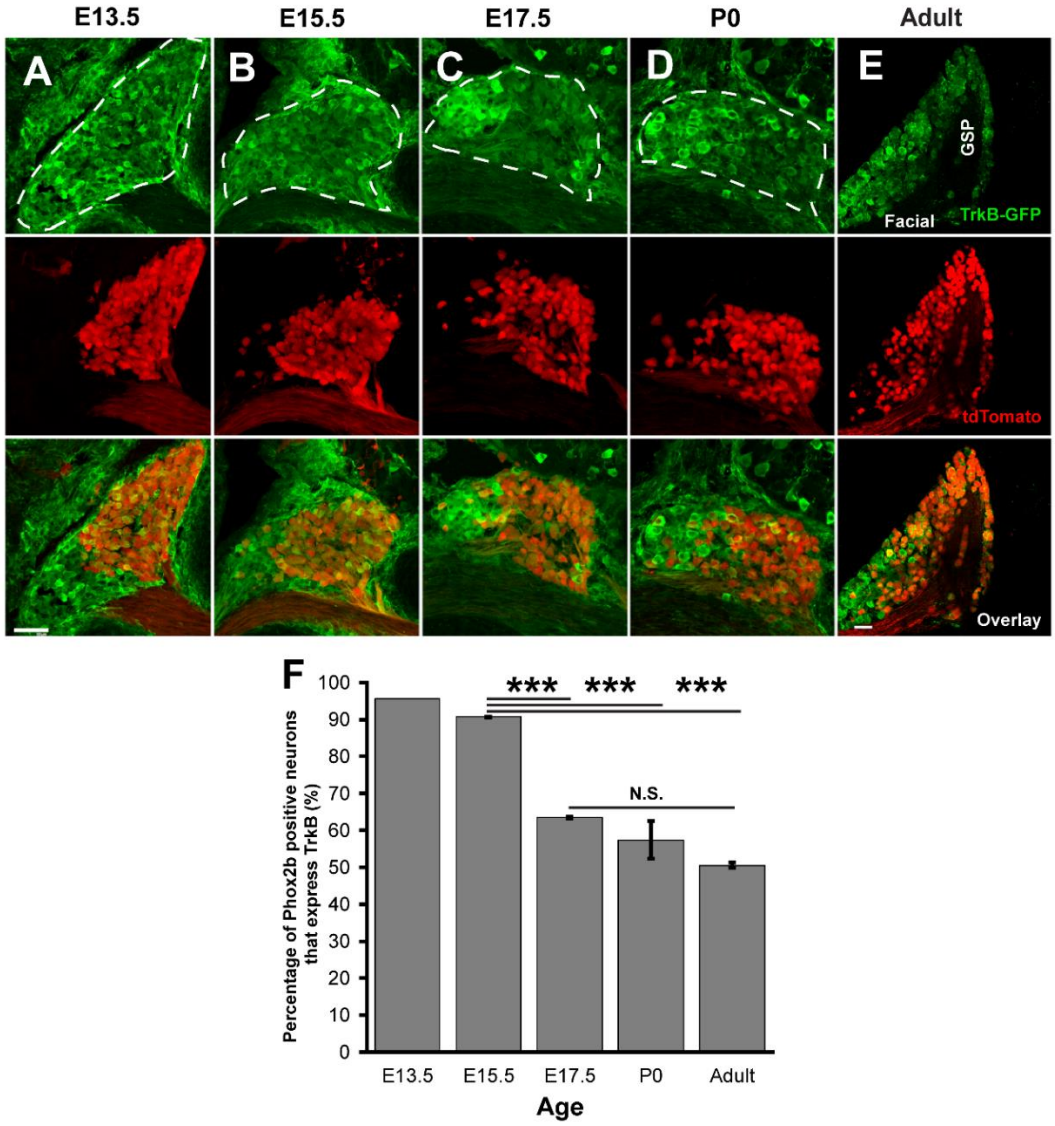

Figure 2.2**. TrkB expression decreases before birth in oral cavity-projecting neurons and remains reduced through adulthood.** A-E) Representative Z-stack images of TrkB-GFP (green, top) and Phox2b-tdTomato (red, middle) from geniculate ganglion sections across ages E13.5 (n = 2), E15.5 (n = 3), E17.5 (n = 4), P0 (n = 3), and P60 (n = 3). Dashed lines illustrate the boundaries of the geniculate ganglion. F) Mean percentage ± SEM of Phox2b-positive neurons that express TrkB receptor during development and adulthood. ***p < 0.001, Scale bar = 50 µm.

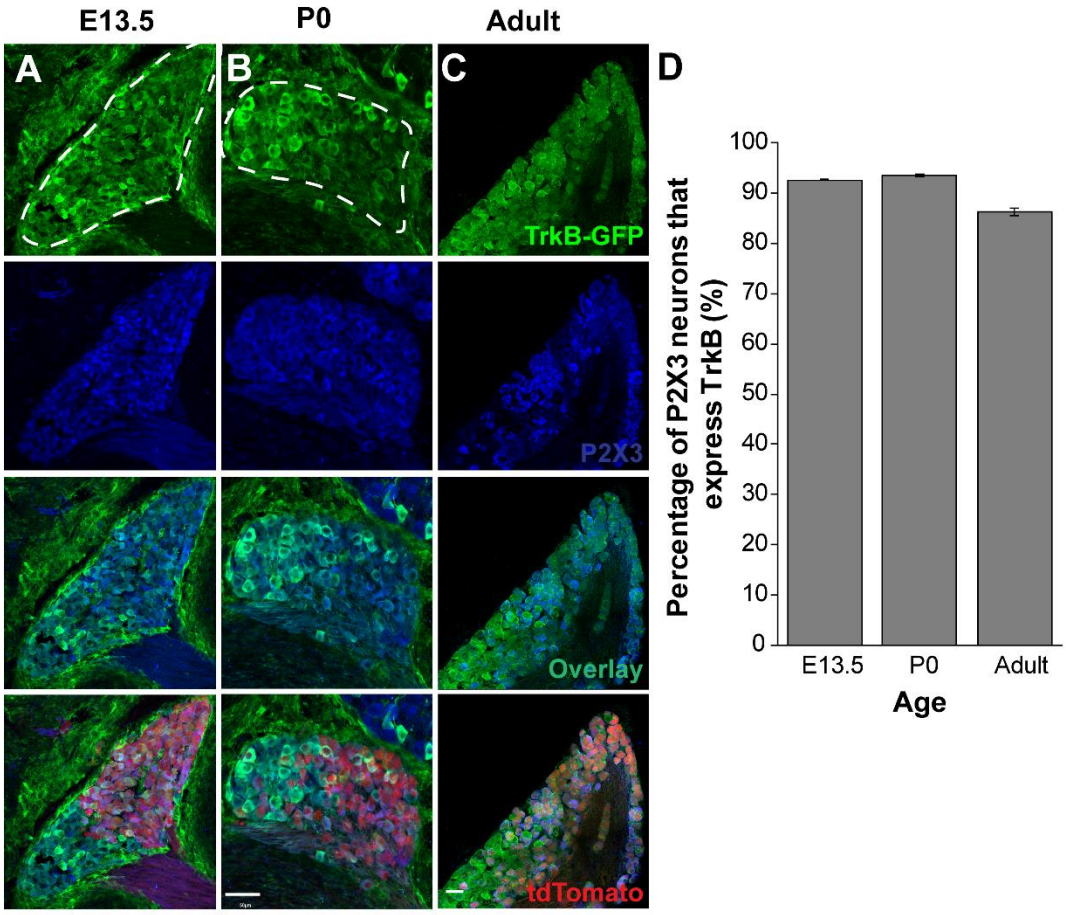

Figure 2.3. **TrkB expression is consistent across ages in non-taste neurons**. A) Representative image of geniculate ganglion sections labeled with TrkB^{GFP} (green), P2X3 (blue) and Phox2b-tdTomato (red) across ages (E13.5 (n=2), P0 (n=3), adult (n=3)). B) Mean percentage ± SEM of non-taste neurons (Phox2b-, P2X3+) that express TrkB during development and adulthood. Scale bar = 50 µm.

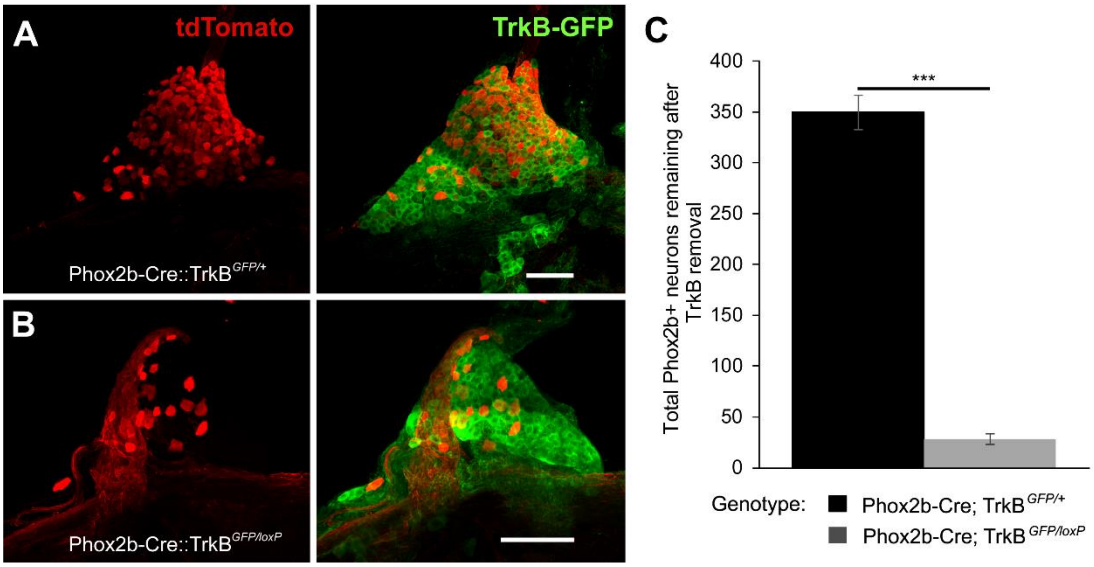

Figure 2.4. **A small population of taste neurons (Phox2b-positive) do not depend on TrkB expression during development.** A) Representative picture of a whole mount of geniculate ganglion at P20 from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ (control) and B) Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ (conditional knockout). C) Mean ± SEM of Phox2b+ neurons remaining after TrkB removal from oral cavity-projecting neurons (control, n = 5; conditional knockout, n = 7 conditional knockout). Both scale bars = 100 µm (A, B). ***p<0.001.

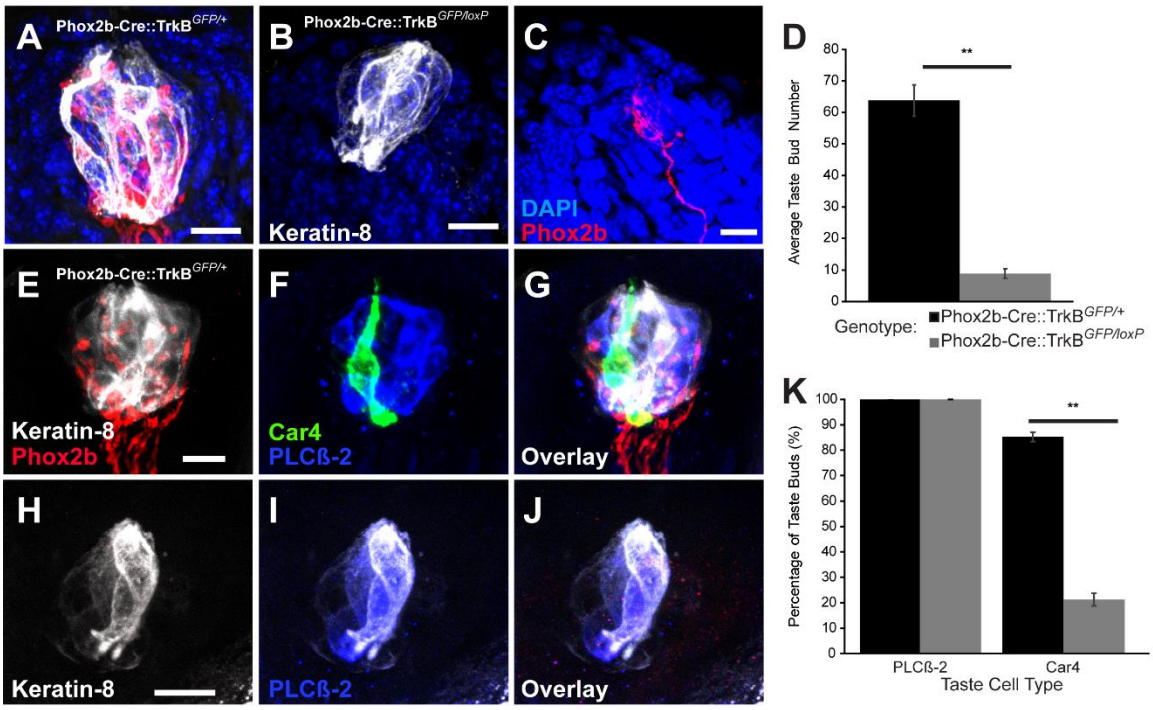

Figure 2.5. **In conditional TrkB knockouts, remaining neurons innervate fungiform papillae, but not taste buds, resulting in substantial taste bud loss by P20.** A) Representative image of a taste bud (keratin-8) with Phox2b+ fibers (tdTomato) from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice. B) Representative image of a taste bud from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ lacking Phox2b- fibers. C) Phox2b+ nerve fiber innervating a fungiform papillae (DAPI). D) Quantification of the number of taste buds remaining after TrkB removal from taste neurons (control, n = 5; conditional knockout, n = 5). E, G) Representative image of Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ taste bud (keratin-8) with innervation (red) from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice, F, G) Car4+ (green) and PLCβ2+ taste receptor cells. H-J) Representative image of Phox2b-Cre::tdTomato::*TrkB*$^{GFP/loxP}$ taste bud (keratin-8) with no innervation from Phox2b-Cre::tdTomato::*TrkB*$^{GFP/+}$ mice and I) PLCβ2+ taste receptor cells K)

Quantification of percentage of taste buds with Car4+ cells. Data represent the mean of percentage ± SEM. Scale bar = 10 μm. Scale bar on E applies to F and G. Scale bar on H applies to I and J. **p<0.01.

2.4. Discussion:

During development, the neurotrophin BDNF and its receptor TrkB play vital roles in the survival and targeting of taste neurons from the geniculate ganglion (Jones, Farinas, Backus, & Reichardt, 1994; L. Ma et al., 2009). Most geniculate neurons express TrkB in the early stages of development (Fei & Krimm, 2013; Yamout et al., 2005), and TrkB is necessary for maintenance of most geniculate neurons at E11.5-E13.5 (Fei & Krimm, 2013; Fritzsch et al., 1997). By adulthood, however, the ligand for TrkB, BDNF, is expressed in a subset of taste receptor cells, and BDNF removal reduces some but not all of the nerve fibers innervating taste buds (Meng et al., 2015; Tang et al., 2017). These findings suggest that by adulthood, TrkB expression may be limited to a subset of taste neurons. Here, I found that not all adult Phox2b+ (taste) neurons expressed TrkB. Specifically, TrkB expression decreased in these neurons between E15.5 and E17.5 such that it was expressed in half the taste neurons by adulthood.

There were two potential explanations for how this developmental reduction in TrkB expression occurred. One possibility was that TrkB receptors were downregulated and became restricted to a subset of geniculate neurons. Alternatively, TrkB expression could have decreased because TrkB- neurons migrate into the geniculate ganglion after E13.5 (Blentic et al., 2011), an age when most geniculate neurons both express and depend on TrkB (Fei & Krimm, 2013). I found that TrkB- neurons do not migrate into the geniculate ganglion at later embryonic ages, and instead the TrkB receptor is downregulated. Specifically, TrkB expression did not decrease until after E15.5, which is later

than might be expected if new TrkB- neurons continue to migrate into the geniculate ganglion. Furthermore, the small number of TrkB- oral cavity neurons present in the E13.5 geniculate ganglion (approximate 5%) accounted for most of the remaining neurons in the conditional TrkB knockout at P20 (approximately 9%). I conclude that most (91%) of the oral cavity neurons initially express and depend on the TrkB receptor and then 41% of these neurons downregulate TrkB between E15.5 and E17.5.

The timing of TrkB downregulation in the oral cavity-projecting neurons corresponds with the timing of decreased BDNF expression in the taste bud (T. Huang & Krimm, 2010). Around E14.5, geniculate neurons depend on BDNF expression in the lingual epithelium to properly innervate their targets (Lopez & Krimm, 2006; L. Ma et al., 2009). By E15.5, geniculate neurons have already reached their targets, so BDNF should no longer be needed in the tongue at such high levels. Also, at these later stages, geniculate neurons no longer depend on BDNF for survival (Hoshino et al., 2010). Thus, the timing of decreased TrkB expression corresponds with changing roles of BDNF-TrkB signaling during development. Another example in these same neurons of a developmentally critical gene changing roles during development, is the growth factor receptor, Ret (27). Ret regulates expression of the transcription factor, Phox2b, embryonically, but later identifies a subset of adult Ret expressing neurons that are likely somatosensory in function (Donnelly et al., 2018). The role of TrkB in adulthood may be to regulate plasticity and branching characteristics

for a subset set of oral sensory geniculate neurons (TrkB-expressing(Tang et al., 2017)), but could also regulate taste function in this same neuron subset.

In the dorsal root ganglion, a series of developmentally expressed transcription factors and growth factor receptors orchestrate neuron subtype development (Lallemend & Ernfors, 2012). However, our current understanding of neuron subtypes and how they differentiate within the geniculate ganglion is still emergent. The transcription factor Phox2b plays a role in the differentiation of neurons that control viscero-sensory functions (D'Autreaux, Coppola, Hirsch, Birchmeier, & Brunet, 2011; Dauger et al., 2003; Pattyn, Morin, Cremer, Goridis, & Brunet, 1997) and so likely specifies oral sensory from articular neurons in the geniculate ganglion (Dvoryanchikov et al., 2017; Ohman-Gault et al., 2017). I found that the TrkB expression is downregulated specifically in this subset of Phox2b+ oral sensory neurons, but not in the auricular neurons. A finding consistent with a higher level of TrkB expression in auricular neurons than oral sensory neurons by adulthood (Dvoryanchikov et al., 2017). Therefore, Phox2b-regulated differentiation into an oral sensory neuron subtype is required for this decrease in TrkB expression. However, this decrease only occurs in half of the Phox2b+ neurons. Given that Phox2b expression first occurs at E9-9.5 (Pattyn et al., 1997; Pla et al., 2008), which is days before the downregulation of TrkB, Phox2b likely initiates a cascade of events orchestrating the differentiation of taste neurons followed by differentiation of neuron subtypes. A complex series of events such that combinations of these factors work together to orchestrate adult expression patterns (24) seems likely. For example, the growth factor receptor,

Ret, is expressed in both TrkB+ and TrkB- neurons, which further divides oral sensory neurons into Ret+TrkB+,Ret+TrkB-,Ret-TrkB+, etc. (Donnelly et al., 2018). Using a combination of factors to specify neuron subtypes permits a smaller number of factors to orchestrate the development of a larger number of neuron subtypes (i.e. two factors can specify four types, etc). It is now clear that multiple molecular subtypes of oral sensory neurons are present in the geniculate ganglion (Dvoryanchikov et al., 2017), although precisely how many is still unclear.

While much of gustatory neuron differentiation may be regulated by a cascade of intrinsically expressed molecular factors, the target also likely regulates gustatory neuron differentiation. Once taste neurons innervate their targets BDNF from the taste bud could maintain TrkB expression in gustatory neurons, such that those innervating BDNF-expressing taste receptor cells retain the higher TrkB levels. Because BDNF is preferentially expressed in the taste receptor cells that express the neurotransmitter serotonin (C. L. Yee et al., 2003), TrkB might be preferentially expressed in neurons that have the serotonin receptor, Htr3a, which mediates the neuronal serotonin response (Larson et al., 2015). Consistently, mean TrkB expression is higher in the Phox2b+neurons that express Htr3a relative to those that do not (Dvoryanchikov et al., 2017).

When TrkB was conditionally removed from Phox2b+ neurons during early development, a few remaining neurons innervated the oral cavity. This is consistent with studies using full TrkB knockouts reporting that TrkB removal substantially reduces the number of geniculate ganglion neurons (Fei & Krimm,

2013; Fritzsch et al., 1997). However, these previous studies could not examine innervation patterns of these remaining neurons, as they lacked appropriate genetic markers. Here, I found a few tdTomato+ fibers innervating the epithelium in a fungiform papilla and no labeled innervation in remaining taste buds. There are several possible sources of these remaining fibers. One is that these few remaining nerve fibers were innervating locations previously occupied by a taste bud. Alternatively, these remaining neurons may be non-taste, and could belong instead to a small somatosensory population in the geniculate ganglion that innervate the oral cavity (Donnelly et al., 2018; Yokota & Bradley, 2016, 2017). This could be the same small population of fewer than 10% of the Phox2b+ geniculate neurons that express somatosensory specific genes, including Mafb (Dvoryanchikov et al., 2017). Consistently, Mafb+ neurons tend to express lower levels of TrkB than other Phox2b+ neurons in the geniculate ganglion (Dvoryanchikov et al., 2017). These remaining neurons could also be a subpopulation supported by GDNF-signaling via the Ret receptor (Donnelly et al., 2018), rather than TrkB.

Consistent with the neuronal loss, condition TrkB knockout mouse tongues had fewer taste buds. Previous reports of full TrkB knockouts indicate that 37% of the taste buds remained (Fei & Krimm, 2013), compared to only 14% in the current study. Furthermore, in one of these earlier studies, 58% of the remaining taste buds were innervated in full TrkB knockout mice (Fei & Krimm, 2013), while I observed no remaining taste bud innervation. One explanation for these discrepancies is that P2X3 was previously used as marker for taste bud

innervation (Fei & Krimm, 2013). P2X3 is expressed in some somatosensory neurons (Staikopoulos, Sessle, Furness, & Jennings, 2007); therefore, non-taste P2X3+ fibers may innervate some of the remaining taste buds. More likely, these two studies examined different postnatal ages; therefore, the remaining Phox2b+ neurons in the geniculate ganglion might initially innervate taste buds at birth but retract by P20, resulting in an additional postnatal taste bud loss.

Taste buds require innervation to retain their normal size and integrity (Guth, 1957; Oakley et al., 1993; H. Sun & Oakley, 2002). Because some taste buds remained despite complete innervation loss by P20, we investigated whether developmental denervation influences some taste cell types more than others. I found that Car4+ taste receptor cells, known to express SNAP25 and have synapses (Lossow et al., 2017; R. Yang et al., 2000), were absent from most remaining taste buds. Alternatively, all remaining taste buds still contained PLCβ2+ taste receptor cells. Therefore, Car4+ taste receptor cells may depend on innervation more than PLCβ2+ receptor cells. Innervation likely supports taste buds at least in part by releasing sonic hedgehog (Castillo-Azofeifa et al., 2017; W. J. Lu et al., 2018). However, in the absence of innervation, the few remaining taste buds could be supported by epithelial-derived sonic hedgehog (Castillo-Azofeifa et al., 2017; Kumari et al., 2015; H. X. Liu, Maccallum, Edwards, Gaffield, & Mistretta, 2004; Miura et al., 2001; Miura, Scott, Harada, & Barlow, 2014).

Taken together, these data show that TrkB expression and dependence divides taste neurons into subpopulations. I suggest that BDNF expression

initially guides TrkB+ fibers to innervate taste buds during a critical developmental period (L. Ma et al., 2009). During this time, all taste neurons express and depend on TrkB, while the 9% of oral cavity-projecting neurons that are TrkB-independent are oral somatosensory. After this critical period, TrkB expression in Phox2b+ neurons is downregulated, dividing these neurons into a TrkB+ and TrkB- subpopulations. In the adult taste system, BDNF likely maintains TrkB expression and taste bud innervation of the TrkB+ but not the TrkB- taste fibers (Tang et al., 2017). These TrkB+ taste neurons may play a different functional role than TrkB- neurons in adulthood. Because taste neuron subclasses are likely defined by combinations of differential gene expression, TrkB likely joins with other factors (Abraira & Ginty, 2013; Donnelly et al., 2018; Dvoryanchikov et al., 2017; Lee et al., 2017) to separate taste neurons into types and influence their ability to innervate subclasses of taste receptor cells

CHAPTER III

BLOCKING TRKB-SIGNALING DISRUPTS TASTE FUNCTION

3.1. Introduction

The neurotrophin, brain derived neurotrophic factor (BDNF), regulates the normal development of the taste system. Specifically, BDNF binding to its receptor, TrkB, causes dimerization and activation of signaling cascades supporting the survival and targeting of taste neurons during development (Hoshino et al., 2010; Krimm et al., 2001; L. Ma et al., 2009; Patel & Krimm, 2010). Both cell death and targeting occur during a critical period of development, after which BDNF is no longer required for either function (Hoshino et al., 2010; L. Ma et al., 2009; Meng et al., 2015). After this critical period, both BDNF and TrkB expression becomes restricted to subsets of taste cells and geniculate neurons, respectively (T. Huang et al., 2015; Tang et al., 2017). This change in expression pattern indicates that BDNF-TrkB signaling likely has new functional roles in the adult taste system.

The role of TrkB signaling in the adult peripheral taste system are unclear, but some possibilities can be gleaned from how BDNF functions in the postnatal and adult central nervous system (CNS). In the CNS, the TrkB signaling pathway plays an important role in both synaptic function and neuron morphology

(Blum & Konnerth, 2005; Galati, Hiester, & Jones, 2016; Park & Poo, 2013; Rauskolb et al., 2010; Rose et al., 2004). BDNF release is activity dependent (H. Lu et al., 2014; Matsuda et al., 2009; Wong et al., 2015) and can regulate neuron function through a variety of mechanisms (Blum & Konnerth, 2005; Chao, 2003), only some of which have likely been identified (Chao, 2003; Rose et al., 2004). For example, application of BDNF induces depolarization within milliseconds via Nav1.9 channels (Blum et al., 2002; Kafitz et al., 1999), influences neuronal excitability via TRPC3 channels (Amaral & Pozzo-Miller, 2007a; H. S. Li et al., 1999), and suppression of Kiv1.3 channels (Mast & Fadool, 2012; Tucker & Fadool, 2002).

In general, peripheral and central roles of BDNF are considered to be fundamentally different (Sasi, Vignoli, Canossa, & Blum, 2017); however, the known peripheral roles are limited to embryonic development, since BDNF continues to be expressed in the adult taste system, we hypothesized that BDNF acquires new roles in adult taste system more similar to its roles in the CNS. Consistently, in the adult taste system, BDNF maintains innervation to the taste bud, without influence on neuron survival (Lopez & Krimm, 2006; L. Ma et al., 2009), likely by regulating dendritic morphology. It is not known if BDNF can regulate taste function independent of morphological changes, as it can in the CNS. However, consistent with the idea that this might be the case, BDNF is expressed in the taste receptor cells with synapses, where it phosphorylates TrkB in the taste bud (I. V. Nosrat, Margolskee, & Nosrat, 2012), making a synaptic role for BDNF possible (T. Huang et al., 2015; C. L. Yee et al., 2003).

The aim of this study was to determine whether TrkB signaling influences taste function. To test this hypothesis, I used a chemical-genetic approach, which allowed TrkB-signaling to be reversibly blocked with 1-NMPP1 in mice with a specific point mutation (*TrkB*^{F616A}) (Chen et al., 2005; Johnson et al., 2008; Y. Liu et al., 2012; Sompol et al., 2011; Vandenberg, Piekarski, Caporale, Munoz-Cuevas, & Wilbrecht, 2015; Wang et al., 2009). I chose this approach because BDNF can have opposing functional roles through different receptors; an excitatory role on function via TrkB (Levine, Dreyfus, Black, & Plummer, 1995), an inhibitory role on function via p75 (Fujita, Takashima, Endo, Takai, & Yamashita, 2011) and truncated TrkB (Eide et al., 1996). Therefore, I expected that blocking only the excitatory role would yield more clear results. I found taste nerve (chorda tympani) responses were reduced to some stimuli, but not others, impacting multiple functional modalities, when TrkB signaling was blocked for 3 hours. I also found behavioral responses were altered to low concentrations of citric acid and these effects were not caused by changes in morphology. These data are consistent with our hypothesis that TrkB signaling influences peripheral adult gustatory system function.

3.2. Material and methods:

3.2.1 Animals

To disrupt TrkB signaling, I used homozygous *TrkB*^{F616A} mice (P40-P70), (RRID: IMSR_JAX:022363). These mice have a single point mutation on the TrkB allele; a substitution of phenylalanine (F) to alanine (A) in the intracellular domain

of TrkB receptor, that allows rapid and reversible blockage of TrkB signaling by application of 1-NMPP1 (Chen et al., 2005; Johnson et al., 2008). Littermates from *TrkB*$^{F616A/+}$ breeding pairs that lacked the point mutation (wild type) were used as control animals for genotype and treatment (electrophysiology recordings and behavior) and to ensure control mice originated from the same background (C57BL6). To examine changes in TrkB fiber morphology after 1-NMPP1 treatment, I crossed *TrkB*$^{Cre\ ER}$::tdTomato mice (RRID:IMSR_JAX:027214) with homozygous *TrkB*^{F616A} mice. The resulting mice (*TrkB*$^{Cre\ ER/F616A}$::tdTomato) express tdTomato following Cre-mediated gene recombination in TrkB-positive neurons. In addition, the TrkB receptor has a binding site for 1-NMPP1, which can be used to block TrkB signaling. All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature, with standard alternating 12-h periods of light and dark and free access to water and food. Animals were cared for and used in accordance with guidelines of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and NIH Guide for the Care and Use of Laboratory Animals.

3.2.2. Tamoxifen administration and 1-NMPP1 injections

TrkB$^{Cre\ ER/F616A}$::tdTomato mice received 4mg/kg of tamoxifen (T5648, Sigma-Aldrich, St. Louis, MO; mixed in peanut oil, 188 ng/g body weight) once per day for 3 days by oral gavage starting at day P39. Three weeks later, adult mice (P60) were injected with 10uL of 200mM 1-NMPP1 (Cayman Chemical,

Cat. No. 221244-14-0) or vehicle (10uL DMSO) s.c. 1-NMPP1 was mixed by dissolving 50mg into 754µL of DMSO to make a 200mM stock solution. Aliquots of 10µl of 200 mM 1-NMPP1 stock solution or 10uL DMSO (vehicle) were diluted into 380µL of injection solution (0.9% NaCl and 2.5% Tween-20) (Johnson et al., 2008). This dose was chosen because it has been used in other studies to inhibit TrkB signaling pathway in a rapid and reversible manner by reducing both TrkB receptor and Erk phosphorylation (Chen et al., 2005; Johnson et al., 2008)

3.2.3. Nerve recordings

To examine the role of BDNF-TrkB signaling on taste responses, I recorded whole nerve taste responses from the chorda tympani (CT) nerve. Briefly, at P40-P70, wild type (vehicle n = 10; 2 males 8 females// 1-NMPP1 n=15; 8 males 6 females,) and *TrkB*^{F616A} (vehicle n=11; 5 males 6 females// 1-NMPP1 n=12; 8 males 4 females) mice were sedated with 0.32 mg/kg intramuscular injection of Domitor (medetomidine hydrochloride, Pfizer Animal Health) and anesthetized with 40 mg/kg intramuscular Ketaset (ketamine hydrochloride, Henry Schein). A water-circulating heating pad was used to maintain body temperature at ~37°C. Bilateral transection of the hypoglossal nerves was performed to prevent tongue movements. Then, the animal was tracheotomized to avoid liquid solutions from entering the lungs. A non-traumatic head-holder was used to position the animal (Erickson, 1966) and a mandibular approach was used to gain access laterally to the chorda tympani nerve near the left tympanic bulla. The nerve was exposed, desheathed, cut and placed on a

platinum-wire electrode (Figure 3.1A). A second electrode was placed on nearby muscle tissues to serve as ground. Kwik-Sil (Cat No. Item#: KWIK-SIL, World Precision Instruments) was placed in the cavity around the nerve. Whole-nerve CT activity was amplified (X10000; A-M Systems, bandpass 300-1500Hz) for all the experiments. Data were recorded and analyzed using Spike2 (RRID:SCR_000903; Cambridge Electronic Design).

3.2.4. Stimulation procedure and data analysis

All the chemicals were reagent grade and prepared fresh in deionized water at room temperature: 20 mM citric acid, 10 mN HCl, 50 mM quinine, 50 mM denatonium, 1 M sucrose, 64 mM acesulfame K^+ (AceK), 100 mM Na-saccharin (Na-sac), 300 mM monosodium glutamate (MSG), 100 and 500 mM NaCl and 100 mM and 500 mM NH_4Cl. The stimuli were applied to the tongue with a syringe for 30-40s (Figure 3.1C, blue arrows). After each chemical stimulation, the tongue was rinsed with deionized water for approximately 60-90s between successive stimulations to bring nerve activity to baseline (Figure 3.1B and 3.1C; black arrows).

Data analysis for relative responses: The amplitude of the integrated responses was measured 10s after onset, to exclude 'somatosensory responses' (phasic phase), for 20s during the steady-state (tonic phase) and averaged for each stimulus. Response amplitudes were plotted as ratios relative to the average response of 100 mM NH_4Cl before and after stimulation. For a taste-evoked response to be considered for relative responses analysis, the two

presentations of 100 mM NH_4Cl could not differ more than 15%. Each individual concentration was compared between vehicle and 1-NMPP1 using Mann-Whitney U test (*p< 0.05) was considered significant and corrected with Bonferroni's for multiple comparisons.

Data analysis for normalized to baseline responses: Using custom written Matlab scripts (RRID: SCR_001622) stimulus onset was determined using change point detection and included 15s pre-stimulus and 30s post-stimulus (red portion of the trace, Figure 3.1B). To control for recording stability, each stimulus delivery was divided into a 15s pre-stimulus and 30s post-stimulus trial window. The baseline for each trial (first 10 seconds) was averaged for each mouse, and trials with baseline shifts more than two standard deviations from the mean were considered unstable and were excluded from analysis. Next, each trial a peri-stimulus time histogram over the 45 second window was generated by dividing every 2.5s of the responses by the average of the baseline. Each peri-stimulus time histogram represents the mean of the normalized response (solid line) and standard error of the mean (dash lines) per group (Figure 3.1D).

Responses to each taste stimulus were averaged across experimental groups. Each taste stimulus averaged normalized response was compared over time between *TrkB*^{F616A} (vehicle vs. 1-NMPP1) using Wilcoxian rank-sum test. Three consecutive bins (7.5s or more) were considered significantly different and correction for FEW requiring at least 3 consecutive bins (black line present *p<0.05). The mean of the last 20s of the responses per stimulus was compared between *TrkB*^{F616A} (vehicle vs 1-NMPP1) using Mann–Whitney U test and

corrected with Bonferroni's for multiple comparisons (*p < 0.05) using Sigma Plot version 14 software (RRID: SCR_003210; Systat Software Inc., San Jose, USA).

3.2.5. Behavioral paradigm

To examine the role of BDNF-TrkB signaling on short-term taste behavior, I used a brief-access task while blocking TrkB signaling. To examine taste function, I trained mice to avoid 10 mM citric acid with LiCl pairings after conditioning sessions. Mice that acquired a conditioned taste aversion, will not only avoid the concentration used for pairings, but will generalize it to other concentrations in a range (Ishiwatari & Bachmanov, 2009; Treesukosol, Mathes, & Spector, 2011). Conditioned taste aversion to citric acid and generalization of this pairing to lower concentration, I paired 10 mM citric acid with either LiCl injections or NaCl (as a control for LiCl), (Table 1) (Ishiwatari & Bachmanov, 2009; Treesukosol et al., 2011). To determine if blocking TrkB-signaling influenced sampling of citric acid or the number of trials initiated, I trained *TrkB*^{F616A} mice using the conditioned taste aversion paradigm.

Water training. Water bottles were removed from home cages on day 0. Mice were trained in the morning (9a-12p) for 15 mins in a multi-channel gustometer (Davis MS-160-Mouse gustometer; DiLog Instruments Inc. *Tallahassee Fl;* (Smith, 2001)). The first 3 days (day 1-3), mice were trained to drink from a stationary spout. Days 4-6, mice that drank from the stationary spout were trained to drink from spouts that moved randomly. Mice could initiate from 40 to 72 trials during the 15min session. For each trial, the shutter remained

open for 5s after the first lick or for a maximum of 10s if the mouse did not initiate a trial. Each trial was followed by a 7.5s intertrial interval during which time a motor changed tubes randomly. Trial duration was consistent during training, conditioning, recovery and testing stages. To prevent dehydration during training sessions, mice had access to a 1mL of water in their home-cage for 1.5 mins in the afternoon approximately 5 hours after the morning training session.

Conditioned taste aversion training. Following water training, wild type mice were induced to conditioned taste aversion by pairing LiCl injections (6.0 mEq/kg, 0.15M; *n* = 6) or NaCl (6.0 mEq/kg, 0.15 M; *n* = 7) immediately after receiving 10 mM citric acid (conditioned stimulus (CS)) in the gustometer. Mice received a total of four pairings of 10 mM citric acid with LiCl or NaCl (D7, D10, D13 and, D16). Each conditioning day was separated by 2 recovery days in which mice received only water during their morning session to reacclimate them to the procedure. For *TrkB*^{F616A} mice, the same CTA protocol was used; pairing LiCl injections with 10 mM CA were used (Table 1).

Behavioral testing. Each wild type mouse was tested in the gustometer with 8 different citric acid concentrations (0.03 mM, 0.1 mM, 0.3 mM, 0.7 mM, 1 mM, 3 mM, 7 mM, 10 mM) with each concentration presented in a different random order for each mouse, except that water was always the first trial. Because lick rates were almost completely suppressed across a broad range of citric acid concentrations in wild type mice receiving conditioned taste aversion, *TrkB*^{F616A} mice were tested with slightly different citric acid concentrations (which included 0.003 mM, and 0.01 mM, instead of 0.7 mM, and 7 mM) with water as

first trial. On test day (day 19), *TrkB*^{F616A} mice received either vehicle or 1-NMPP1 3 hours prior to the testing procedure.

Data analysis. For each stimulus, the total number of licks and trials engaged were measured for water and the different concentrations of citric acid during the conditioned taste aversion retention test. Total licks to different concentrations of citric acid relative to water were compared between treatment (wild type: LiCl vs NaCl; *TrkB*^{F616A}: vehicle vs 1-NMPP1) using two-way ANOVA, followed by Tukey's HSD test as post hoc; *p< 0.05 was considered significant. The number of trials engaged for each citric acid presentations relative to water were compared between treatments in *TrkB*^{F616A} mice that received vehicle versus 1-NMPP1 using Student t-test, *p< 0.05 was considered significant.

3.2.6. Immunohistochemistry

Adult *TrkB*$^{Cre\ ER/F616A}$:: tdTomato mice (P60) were sacrificed 3 hours after 1-NMPP1 or vehicle injections by with an overdose (1mL) of 2.5% tribromoethanol (Avertin) i.p., and trans-cardially perfused with 4% paraformaldehyde (PFA). Tongues were dissected and post-fixed in 4% PFA overnight at 4°C. Tissue was transferred to 30% sucrose/PBS for cryoprotection at 4°C overnight. The following day, the tongue was cut in half at the midline, frozen on dry ice in optimum cutting temperature (O.C.T.) embedding medium and stored at -80C until immunohistochemistry. For thick sections, tongue halves were sectioned at 70µm and collected in 0.1M PB. The sections were rinsed in 0.1M PB tissue and then blocked for 2 days with 3% donkey serum in 0.1M

phosphate-buffer (PB) containing 0.5% Triton X-100 at 4°C. To visualize TrkB-positive nerve fibers within taste buds, thick sections were incubated with the following primary antibodies for 5 days at 4 °C: rabbit polyclonal anti-dsRed (1:500, Clontech Laboratories, Inc., RRID: AB_10013483 Cat No. 632496, Mountain View, CA) and rat anti-cytokeratin 8 in PBS (1:50; Developmental Studies Hybridoma Bank, RRID: AB_531826, Cat. No. Troma-1-s, Iowa City, IA). After incubation in primary antibodies and four rinses in 0.1M PB, tissues were incubated in the following secondary antibodies for two days: goat anti-rat Alexa-Fluor 488 (1:500; Jackson ImmunoResearch Laboratories) and goat anti-rabbit Alexa Fluor 555 (1:500; Jackson ImmunoResearch Laboratories). The tissue was then washed four times in 0.1 M PB, mounted onto slides, and cover-slipped using aqueous mounting medium (Fluoromount-G, Southern Biotech, Birmingham, AL). To visualize whole taste buds and quantify branching characteristics, I performed whole mount staining of the entire lingual epithelium on the remaining tongue halves, as previously described (Ohman-Gault et al., 2017). Briefly, the lingual epithelium was separated from most of the underlying muscle using surgical scissors under a dissecting microscope. The epithelium was then frozen flat in OCT and the remaining muscle was shaved off using a cryostat. Then, the lingual epithelium was cut into smaller pieces (tip and mid portions of the fungiform field) and processed for immunohistochemistry using the same protocol as described above for thick sections.

3.2.7. Quantification of TrkB fiber innervation and branching characteristics within the taste buds.

Since gene recombination in TrkB-positive fibers induced by tamoxifen occurs randomly, only a subset of TrkB-positive fibers will be labeled with tdTomato. Also, the TrkB receptor is expressed in epithelium including taste bud cells causing a few of them to become labeled also. I used a small dose of tamoxifen in order to sparsely label nerve fibers. I injected the tamoxifen, 3 weeks before euthanizing the mice, because taste labeled receptor cells have died and left the bud by then, and nerve fibers are completely filled with tdTomato. The numbers of taste buds innervated by tdTomato labeled nerve fibers (a subset of TrkB-positive fibers) were counted in thick sections of the tongue using a Leica DMLB microscope. The total number of taste buds with tdTomato labeled fibers was compared between mice that received 1-NMPP1 versus vehicle 3 hours prior perfusion using t-test followed by Tukey's HSD test as post hoc; *p <0.05 was considered significant. Taste buds were imaged from the tip of the tongue where taste bud density is higher using Olympus Fluoview FV1200 Laser scanning confocal microscope. Optical images were captured every 0.47μm with a 60× oil objective at a zoom level of 4x. For each image, each channel was taken separately using single-wavelength excitation and merged to produce a composite image. Four to six taste buds with tdTomato labeled fibers per mouse were used for analysis. All the taste bud images were deconvoluted using Deconvolution Software (RRID:SCR_002465 AutoQuant X, Media Cybernetics, MD, USA). Each labeled fiber was traced as it entered the

taste bud (defined by cytokeratin-8 labeling) using Neurolucida 360 software (MBF Bioscience, Williston, Vermont, USA). The number of fibers entering the taste bud, fiber length, number of branch points, and branch ends were measured.

3.3. Results:

3.3.1. Blocking TrkB-signaling reduced chorda tympani responses to some taste stimuli

Although in the central nervous system, BDNF acts as a neuromodulator, it is unknown whether BDNF-TrkB signaling influences adult taste system function in adulthood. To test this idea, I used a chemical-genetic approach, which allowed TrkB-signaling to be reversibly blocked by 1-NMPP1 in mice with a specific point mutation in the intracellular domain of the TrkB receptor (*TrkB*^{F616A}). Three hours after 1-NMPP1 injections, I recorded the whole nerve responses of chorda tympani nerve to the following taste stimuli: 100 mM and 500 mM NH_4Cl, 100 and 500 mM NaCl, 64 mM acesulfame K^+, 100 mM Na-saccharin, 1 M sucrose, 300 mM monosodium glutamate (MSG), 50 mM quinine-HCl, 50 mM denatonium, 20 mM citric acid, and 10 mN HCl. Figure 3.2 shows representative integrated whole nerve responses to different taste stimuli in wild type mice injected with either vehicle (Figure 3.2A) or 1-NMPP1 (Figure 3.2B), and *TrkB*^{F616A} mice injected with vehicle (Figure 3.2C) or 1-NMPP1 (Figure 3.2D). Each representative raw trace was selected because its height was the closest to

the median height of the responses for that stimulus/group. The magnitude of responses in *TrkB*^{F616A} mice with 1-NMPP1 for 3 hours (red traces on Figure 3.2D) showed a general suppression compared to wild type with vehicle, wild type with 1-NMPP1, and *TrkB*^{F616A} with vehicle (black traces Figure 3.2A,B & C respectively).

To determine whether TrkB signaling influences taste function, I measured the amplitude of taste responses per stimulus averaged over a 20s stimulation window (tonic phase) for each group (Figure 3.3A-E). The magnitudes of the taste responses were not significantly different in wild type mice that received 1-NMPP1 compared to vehicle (Figure 3.3). However, I found a general suppression in the magnitude of taste responses to most of the stimuli in *TrkB*^{F616A} mice with 1-NMPP1 compared to *TrkB*^{F616A} mice that received vehicle.

Sour responses to both citric acid and HCl were significantly reduced in *TrkB*^{F616A} mice with 1-NMPP1 compared vehicle (Figure 3.3A, vehicle vs 1-NMPP1; citric acid: **p = 0.004, HCl: **p = 0.003). Bitter responses to quinine and to denatonium were not significantly reduced in *TrkB*^{F616A} mice compared to *TrkB*^{F616A} mice that received vehicle (Figure 3.3B, quinine-HCl: p = 0.05; denatonium: p>0.05). Sweet responses to sucrose, acesulfame K^+ and Na-saccharin were not significantly reduced in *TrkB*^{F616A} mice treated with 1-NMPP1 compared to vehicle (Figure 3.3C; sucrose: p >0.05, acesulfame K^+: p>0.05 and Na-saccharin: p > 0.05). Umami responses to monosodium glutamate were significantly reduced in *TrkB*^{F616A} mice with 1-NMPP1 compared vehicle (Figure 3.3D; MSG, *p =0.02). Salt responses to 500 mM NaCl and 100 mM, 500 mM

NH_4Cl were significantly reduced (Figure 3.3E: 500 mM NaCl, *p = 0.02; 100 mM and 500 mM NH_4Cl, **p =0.01). These data indicate that blocking TrkB signaling for 3 hours prior recording reduced the amplitude of the taste responses to different taste stimuli in *TrkB*^{F616A} mice.

3.3.2. Responses to taste stimuli were not significantly different when plotted relative to 100 mM NH_4Cl.

Applications of NH_4Cl in brackets before and after a series of taste stimulations are used to monitor nerve viability and differences between animals. It is also the case that taste responses are typically reported relative to the average of NH_4Cl application since changes in the height of NH_4Cl are frequently used as indicator of nerve stability during a recording session (Breza & Contreras, 2012a, 2012b; Inoue et al., 2007; Skyberg et al., 2017; C. Sun et al., 2015; C. Sun et al., 2017; C. Sun, Krimm, & Hill, 2018). Therefore, I first analyzed our data using this approach. Responses between two NH_4Cl responses in which change less than 15% before and after, were considered unstable and were excluded from analysis. These remaining responses were plotted relative to 100 mM NH_4Cl (Feng et al., 2015; T. M. Nelson et al., 2010; Qin et al., 2018; Shigemura et al., 2013).

I found that relative taste responses to 100 mM NH_4Cl, were not different across genotype and treatment for sours (Figure 3.4A; citric acid: p >0.05), bitters (Figure 3.4B; quinine-HCl: p >0.05; denatonium: p > 0.05), sweets (Figure 3.4C; sucrose: p >0.05 ; acesulfame K^+: p >0.05; Na-saccharin: p >0.05 and salts

(Figure 3.4E; 500 mM NaCl: p >0.05; 500 mM NH_4Cl: p >0.05). Yet, relative responses to HCl in TrkB^{F616A} mice were significantly reduced (TrkB vehicle vs 1-NMPP1, p > HCl: * p = 0.01). Oddly, relative responses to monosodium glutamate (MSG) in wild type with 1-NMPP1 were significantly reduced but not in *TrkB*^{F616A} mice (Figure 3.4D; wild type vehicle vs 1-NMPP1, **p =0.01; TrkB vehicle vs 1-NMPP1, p >0.05). It was possible that responses to 100 mM NH_4Cl were reduced to the same degree as the responses to other stimuli, such that the relative responses yielded no effect.

3.3.3. **Normalized responses were significantly to different taste stimuli when plotted relative to baseline.**

Since I speculated that plotting responses relative to NH_4Cl masked the general suppression observed from the raw traces, I sought an alternate method of analysis. In some taste studies where responses to NH_4Cl were affected, taste responses were normalized to baseline (5 seconds before taste stimulation) (Larson et al., 2015; Vandenbeuch et al., 2013). I decided to use a similar approach to normalize the amplitude of the integrated responses relative to their baseline (pre-stimulus). However, it was unclear from previous studies how nerve viability and recording stability over time was monitored. To address recording stability, chorda tympani responses to different stimuli were sorted as trials. For each animal, trials with a shift of two standard deviations in the baseline from the mean were considered unstable and were excluded from data analysis. Additionally, I generated peri-stimulus time histograms for each stimulus, which

illustrates both the mean and variability for each group response aligned over a 45s window (peri-stimulus time histograms in Figure 3.1D).

This analysis allowed me to compare taste responses over time between mice treated with 1-NMPP1 and mice with vehicle. To determine significant temporal differences in taste responses over the progression of the evoked response (taste x time), I compared each 2.5s over a 30s window which included the transient phasic phase (first 10s) and the steady-state or tonic phase (20s following the phasic phase). I found that sour responses to citric acid in *TrkB*^{F616A} mice treated with 1-NMPP1 compared to vehicle were significantly reduced over time (Figure 3.5A black bar, 7.5s-17.5s; Wilcoxian rank-sum, * $p<0.05$). Salt responses to NH_4Cl were significantly reduced in *TrkB*^{F616A} receiving 1-NMPP1 compared to vehicle over time, but only for high concentrations (Figure 3.5L: black bar, 2.5s-30s; rank sum, *$p<0.05$). Normalized responses to quinine-HCl in *TrkB*^{F616A} 1-NMPP1 group compared to vehicle were significantly reduced over time (Figure 3.5C black bar, 7.5s-17.5s; *$p<0.05$). Sweet responses to acesulfame K^+ were significantly reduced in *TrkB*^{F616A} 1-NMPP1 group compared to vehicle over time (Figure 3.5F black bar, 7.5s-30s; rank sum, *$p<0.05$).

In addition, when I examined the average of the last 20s (tonic phase) of the normalized sour responses in *TrkB*^{F616A} mice treated with 1-NMPP1 compared to vehicle, responses to both citric acid (Figure 3.5A bar graph: * $p = 0.04$) and HCl (Figure 3.5B bar graph: * $p =0.03$) were significantly reduced. Sour was the taste quality where responses to multiple stimuli were reduced by blocking TrkB-signaling. Normalized responses to quinine-HCl were also reduced

in *TrkB*^{F616A} 1-NMPP1 group compared to *TrkB*^{F616A} with vehicle (Figure 3.5C bar graph: *p = 0.023) when the mean of the last 20s was compared. Umami responses were altered in *TrkB*^{F616A} with 1-NMPP1 group compared to *TrkB*^{F616A} with vehicle (Figure 3.5H bar graph: * p =0.05).

Sweet responses to acesulfame K^+ were significantly reduced in *TrkB*^{F616A} mice with 1-NMPP1 compared to *TrkB*^{F616A} with vehicle (Figure 3.5F bar graph: * p = 0.02). Yet, sweet responses to sucrose and Na-saccharin were not different in *TrkB*^{F616A} with 1-NMPP1 group compared to *TrkB*^{F616A} with vehicle (Figure 3.5E bar graph: sucrose: p >0.05; Figure 3.5G bar graph) Na-saccharin: p >0.05).

Salt responses to low and high concentrations of NaCl were not significantly different in *TrkB*^{F616A} with 1-NMPP1 group compared to *TrkB*^{F616A} with vehicle (Figure 3.5I bar graph: 100 mM NaCl: p >0.05; Figure 3.5J bar graph: 500 mM NaCl: p >0.05). Conversely, salt responses to low (100 mM) and high (500 mM) concentrations of NH_4Cl were significantly reduced in *TrkB*^{F616A} with 1-NMPP1 group compared to *TrkB*^{F616A} with vehicle (Figure 3.5K bar graph: 100 mM NH_4Cl: *p = 0.03; Figure 3.5L bar graph: 500 mM NH_4Cl: *p = 0.02).

I found that normalized responses to most stimuli were not significantly different in wild type mice that received 1-NMPP1 compared to vehicle (citric acid: wild type vehicle 2.77±0.16 vs. wild type 1-NMPP1 2.45±0.24, p >0.05; HCl: wild type vehicle 2.44±0.21 vs. wild type 1-NMPP1 2.39±0.23, p >0.05; quinine-HCl: wild type vehicle 1.80±0.16 vs. wild type 1-NMPP1 1.54±0.08, p >0.05; sucrose: wild type vehicle 2.14±0.27 vs. wild type 1-NMPP1 1.93±0.24, p>0.05; acesulfame K^+: wild type vehicle 1.67±0.21 vs. wild type 1-NMPP1 1.56±0.14,

p >0.05; Na-saccharin: wild type vehicle 1.79±0.08 vs. wild type 1-NMPP1 1.68±0.13, p >0.05; MSG: wild type vehicle 1.82±0.12 vs wild type 1-NMPP1 1.61±0.12, p >0.05; 100 mM NaCl: wild type vehicle 1.62±0.10 vs..05; MSG: wild type vehicle 1.82±0.12 vs. wild type 1-NMPP1 1.50±0.11, p >0.05; 100 mM NH_4Cl: wild type vehicle 1.85±0.07 vs. wild type 1-NMPP1 1.80±0.12, p >0.05; 500 mM NH_4Cl: wild type vehicle 3.73±0.24 vs. wild type 1-NMPP1 3.26±0.33, p >0.05). However, normalized responses to denatonium and 500 mM NaCl were significantly different in wild type mice with 1-NMPP1 compared to vehicle (denatonium: wild type vehicle 1.64±0.10 vs. wild type 1-NMPP1 1.23±0.07, *p =0.011 and 500 mM NaCl: wild type vehicle 2.89±0.22 vs. wild type 1-NMPP1 2.41±0.20, * p = 0.047).

In conclusion, when I normalized responses to baseline, our data indicate that TrkB signaling is required for normal responses to sours, some artificial sweeteners, bitters and salts. These effects were absent when responses were plotted as ratios to NH_4Cl (reference stimulus). I was able to use this method to confirm recording stability and compare responses across time (30s). Using this method, I found that blocking TrkB-signaling reduced most taste responses (last 20s of response). However, only taste responses to sour were reduced to both stimuli tested (citric acid and HCl).

3.3.4. Blocking TrkB signaling disrupts citric acid detection using conditioned taste aversion in a brief-access test.

Given that our electrophysiological results showed that responses to all tested sour taste stimuli were suppressed by disrupting TrkB signaling, I aimed to examine the functional role of TrkB signaling in sour taste using a behavioral assay. I chose to focus on citric acid because out of the two sour solutions tested in chorda tympani nerve recordings, citric acid was reduced when plotted as the mean of the 20s of the tonic phase and showed a significant reduction over time (black bar, 10s; Figure 3.5A). Therefore, under normal conditions, mice will drink large amounts of different concentrations of citric acid. I designed a behavioral paradigm in which mice are motivated to avoid drinking citric acid at low concentrations in a brief-access test using conditioning taste aversion (CTA). When mice learn an association between 10mM citric acid and LiCl injections (gastro intestinal malaise), they avoid 10 mM citric acid and generalize their behavior to other citric acid concentrations (Ishiwatari & Bachmanov, 2009; Treesukosol et al., 2011). To verify the protocol, wild type mice had access to 10 mM citric acid, and immediately after received LiCl injections or NaCl (as control) (refer to Table 1; conditioning). After four conditioning pairings, the number of licks to 0.1-10 mM citric acid relative to water were significantly reduced in wild type mice that received LiCl injections compared to those injected with NaCl (Figure 3.6B, *$p<0.05$). These results confirmed that our conditioned taste aversion protocol was effective for mice to learn to avoid concentrations of citric acid that they would typically drink at levels comparable to water in the absence of a conditioned taste aversion.

Using the conditioned taste aversion protocol, I trained *TrkB*^{F616A} mice to avoid 10 mM CA (Table 1). I found that after the third (CS3) and the fourth (CS4) pairing of 10 mM citric acid with LiCl injections, the number of trials engaged and mean licks were significantly reduced after the third (CS3) and the fourth (CS4) during conditioning in *TrkB*^{F616A} mice (Figure 3.6C, **p<0.01 and 3.6D, **p<0.01). These data confirmed that *TrkB*^{F616A} mice learned an aversive association to 10 mM citric acid. Animals that failed to learn the aversive association to citric acid after their fourth pairing were removed (n = 3) . When I tested a range of citric acid concentrations compared to water (Day19), *TrkB*^{F616A} mice that received vehicle 3 hours prior to the task had significantly fewer licks to 0.003 mM, 1 mM, 3 mM and 10 mM citric acid when compared to water (Figure 3.6E, black line, *p <0.05). These findings suggest that *TrkB*^{F616A} mice that received vehicle associated the taste aversion to not only 10 mM citric acid but also generalized it to lower citric acid concentrations. *TrkB*^{F616A} mice with 1-NMPP1 had significantly fewer licks at the higher concentrations (1 mM, 3 mM, 10 mM) of citric acid compared to water (Figure 3.6E, red line, p<0.05) indicating that learning was generalized to high, but now lower concentrations of citric acid. In addition, *TrkB*^{F616A} mice that received 1-NMPP1 licked significantly more both to 0.003 mM and 0.03 mM of citric acid compared to *TrkB*^{F616A} mice that received vehicle injections 3 hours prior (Figure 3.6E). These data suggest that blocking TrkB signaling altered *TrkB*^{F616A} mice taste aversion generalization to lower concentration of citric acid.

Since *TrkB*^{F616A} mice that received 1-NMPP1 could not detect citric acid at low concentrations, I examined whether this finding was because *TrkB*^{F616A} mice initiated more trials when treated with 1-NMPP1 compared to vehicle. I compared the number of trials engaged to the number of trials available per concentration of citric acid. I found no significant differences in the number of trials initiated at each concentration by *TrkB*^{F616A} mice that received vehicle compared to 1-NMPP1, indicating that increased licking was not due to the initiation of more trials at low citric acid concentrations. Interestingly, when all of these trials were combined, *TrkB*^{F616A} mice that received vehicle engaged significantly fewer trials overall to citric acid solutions compared to 1-NMPP1 (Figure 3.6F, bar graph total CA: *$p<0.05$). These findings indicate that *TrkB*^{F616A} with vehicle were less motivated to drink citric acid solutions in general, perhaps due their greater similarity to the CS for these mice compared with those that received 1-NMPP1. Overall, these data suggest that disruption of TrkB signaling can disrupt detection of a conditioned taste aversion to low concentrations of citric acid on a brief-access test.

3.3.5. Disrupting TrkB-signaling for 3 hours with 1-NMPP1 had no effect on branching characteristics of TrkB afferent fibers

Taste bud innervation by TrkB fibers is reduced after removing BDNF from the lingual epithelium (Meng et al., 2015; Tang et al., 2017) suggesting that BDNF sustains taste bud innervation in adulthood. Long term effects of the TrkB signaling pathway can modify dendritic spines and morphology of neurites

(Ekman, Zhu, Sward, & Uvelius, 2017; Ji et al., 2010; Yamout et al., 2005). However, it is unclear whether blocking TrkB signaling in taste fibers could induce morphological changes within the 3-hour period. To determine whether or not the functional changes observed in whole nerve recordings from the chorda tympani nerve and behavioral tests were due to morphological changes in innervation patterns, I examined branching characteristics of TrkB fibers within taste buds. I used a semi-sparse labeling approach in which *TrkB*$^{Cre-ER/F616A}$::tdTomato mice received a dose of tamoxifen (4mg/kg) by oral gavage for 3 days. This induced gene recombination labeling with tdTomato in many but not all TrkB-expressing fibers. If instead, all nerve fibers labeled, more than 50% of the neurons would need to withdraw completely from the taste bud for any taste buds to lose all their innervation achieving a measurable effect (Patel & Krimm, 2010). Furthermore, when all taste nerve fibers are labeled the nerve plexus within the taste bud is too dense to measure separate within the taste bud (Ohman-Gault et al., 2017). This approach permits evaluation of both the number of taste buds innervated by a small randomly selected group of neurons, and evaluation of the branching characteristics of individual fibers in the taste bud. Then, I quantified the number of taste buds innervated by labeled fibers and their branching characteristics. Since any effects of blocking TrkB-signaling are likely to be subtle, I designed this semi-sparse analysis to be more sensitive than traditional approaches.

After 3 weeks of tamoxifen administration, *TrkB*$^{Cre\ ER/F616A}$::tdTomato mice were injected with vehicle or 1-NMPP1 and perfused three hours later for

anatomical analysis (Figure 3.7A). I labeled taste buds with anti-cytokeratin-8 (green) and labeled TrkB fibers with anti-dsdRed (red) (Figure 3.7B). Because the truncated isoform of TrkB receptor is expressed in the epithelium, I found tdTomato label on some perigermal cells (arrows on Figure 3.7B). The percentage of taste buds innervated with tdTomato-labeled fibers was quantified. I found no differences in the percentage of taste buds with TrkB fibers in *TrkB*$^{Cre-ER/F616A}$::tdTomato mice that received 1-NMPP1 compared to vehicle group (1-NMPP1: 41.8±15.6% vs. vehicle: 55.8±17.5%; $p>0.05$). To determine whether blocking TrkB signaling for 3 hours induced any morphological changes in TrkB fibers innervating taste buds in the fungiform field, I examined the branching characteristics of TrkB fibers as they entered the taste bud (Figure 3.7C). I found no differences in the branching characteristics between *TrkB*^{F616A} with 1-NMPP1 compared to vehicle (Figure 3.7D; branch nodes: vehicle 1.7±0.1, 1-NMPP1: 1.4±0.2, $p>0.05$; branch ends: vehicle: 3.0±0.2 vs. 1-NMPP1: 2.4±0.2, $p>0.05$) or length of TrkB fibers (vehicle: 74.4±7.4 vs. 1-NMPP1: 60.2±11.9; $p>0.05$). I did not find any significant changes in nerve fiber length or branching patterns of TrkB-expressing afferent fibers. These data suggest that functional changes observed in chorda tympani recordings and behavioral paradigms were not due to morphological changes on afferent TrkB-fibers innervating the taste buds.

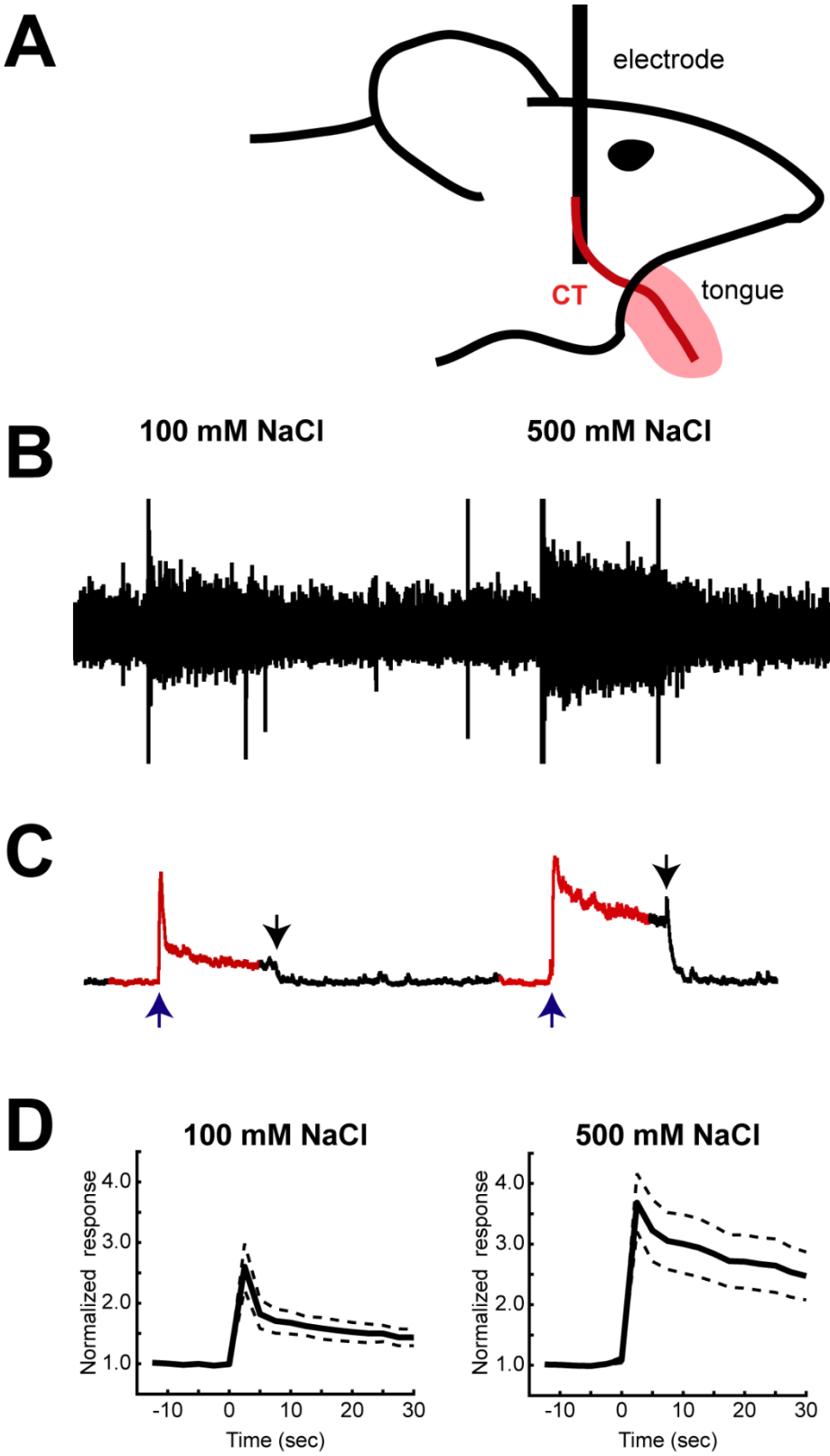
A
electrode
CT
tongue
B
100 mM NaCl
500 mM NaCl
C
D
100 mM NaCl
500 mM NaCl
Normalized response
4.0
3.0
2.0
1.0
-10
0
10
20
30
Time (sec)

Figure 3.1. **Example of raw data and baseline normalized responses from chorda tympani nerve recordings.** A) Whole nerve responses were recorded from the chorda tympani nerve using a mandibular approach. B) A representative trace of not integrated and C) integrated whole nerve responses evoked by 100 mM and 500 mM NaCl stimulation. Amplitude of the evoked response increased with stimulus concentration. Blue arrows indicate the stimulus onset and black arrows represent the initiation of water rinses. Each trial window used for generation of peri-stimulus time histograms consisted of the 15s before and 30s after stimulus delivery (red trace). C) Each trial was normalized to its baseline. A peri-stimulus time histogram represents the average normalized response to a given stimulus. Peri-stimulus time histogram were calculated by normalizing each 2.5 seconds of the response to baseline for each response. Data shown as mean (solid line) ± standard error of the mean (SEM) (dashed lines) over a 45s window. Bin size = 2.5 seconds.

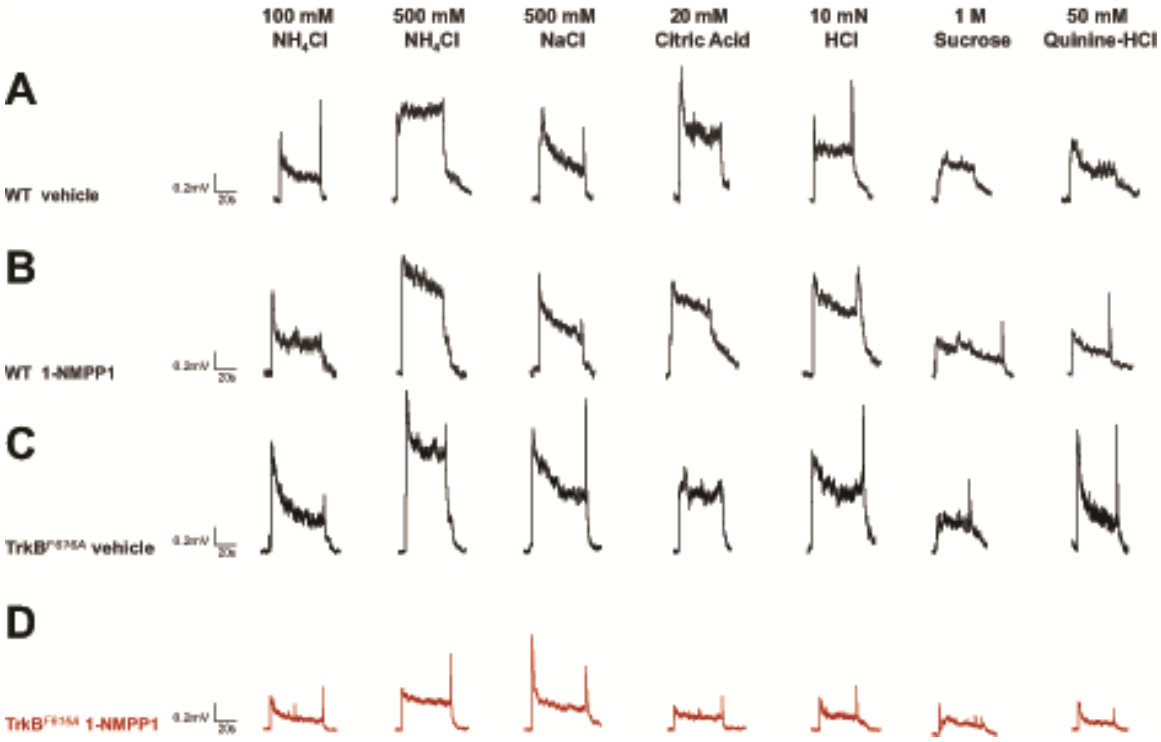

Figure 3.2. **Whole nerve responses to taste stimuli appear reduced in *TrkB*^{F616A} mice injected with 1-NMPP1**. A) Representative integrated chorda tympani nerve responses from wild type vehicle, B) wild type 1-NMPP1, C) *TrkB*^{F616A} vehicle and D) *TrkB*^{F616A} 1-NMPP1 (red) to 100 mM and 500 mM NH_4Cl, 500 mM NaCl, 20 mM citric acid, 10 mN HCl, 1 M sucrose and 50 mM quinine-HCl. Representative traces were selected closest to the median height of the average group response. Each stimulus was applied for ~30s followed by a water rinse for ~60-90s.

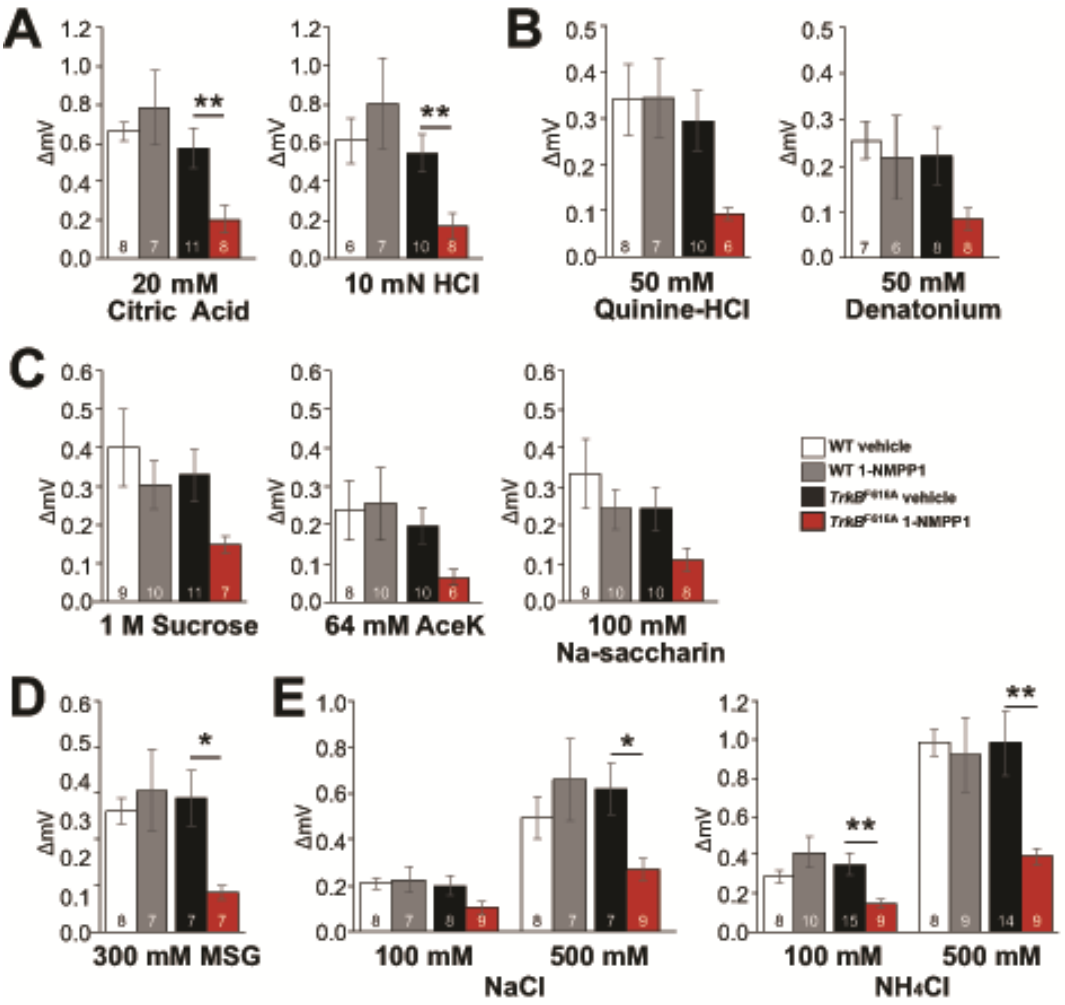

Figure 3.3. **The mean change in amplitude of the integrated whole nerve responses is reduced in *TrkB*^{F616A} with 1-NMPP1.** A-E) Bar graphs show the change in voltage from baseline over the last 20s period of the tonic phase of the response for the four groups of mice: wild type vehicle (open bars), wild type 1-NMPP1 (gray), *TrkB*^{F616A} vehicle (black) and *TrkB*^{F616A} 1-NMPP1 (red). A) Sour stimuli: 20 mM citric acid and 10 mN HCl. B) Bitter stimuli: 50 mM quinine-HCl and 50 mM denatonium. C) Sweet stimuli: 1 M sucrose 1M, 64 mM acesulfame K^+ and 100 mM Na-saccharin. D) Salt stimuli: 100 mM and 500 mM NaCl and 100 mM and 500 mM NH_4Cl. E) Umami stimuli: MSG 300 mM. Figure legend

applies to A-E bar graphs. Data are shown as the mean ± standard error of the mean (SEM). *p<0.05, **p<0.01.

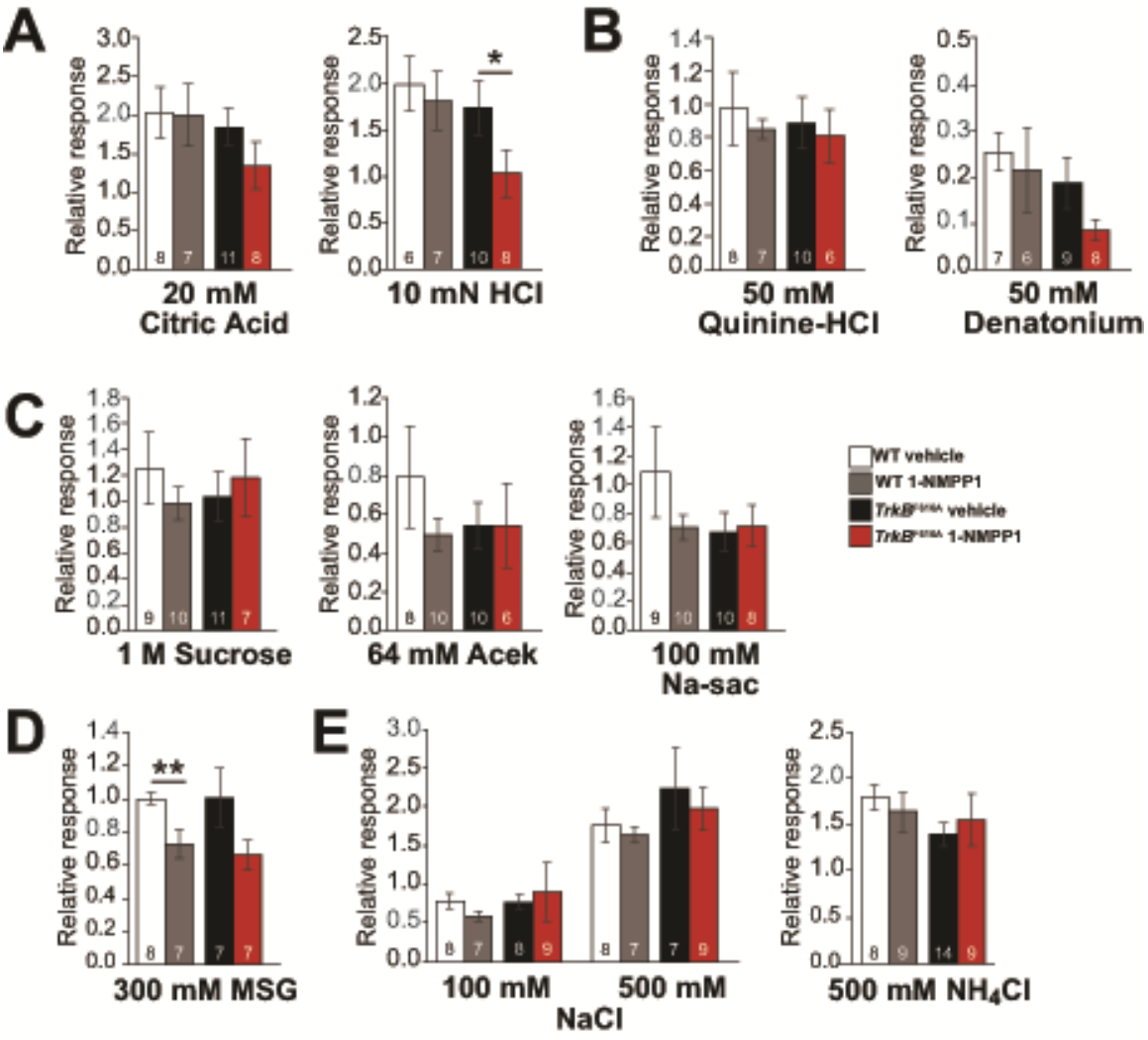

Figure 3.4. **Mean taste responses to 100 mM NH_4Cl were not significantly different.** A-E) Bar graphs represent the mean responses relative to 100 mM NH_4Cl over the last 20s period of the tonic phase for the four groups of mice: wild type vehicle (open bars), wild type 1-NMPP1 (gray), *TrkB*^{F616A} vehicle (black) and *TrkB*^{F616A} 1-NMPP1 (red). A) Sour stimuli: 20 mM citric acid and 10 mN HCl. B) Bitter stimuli: 50 mM quinine-HCl and 50 mM denatonium. C) Sweet stimuli: 1 M sucrose, 64 mM acesulfame K^+ and 100 mM Na-saccharin. D) Salt stimuli: 100 mM and 500 mM NaCl and 500 mM NH_4Cl E) Umami stimuli: 300 mM MSG

responses were significantly different in wild type mice treated with 1-NMPP1. Data shown as mean ± standard error of the mean (SEM). *p<0.05

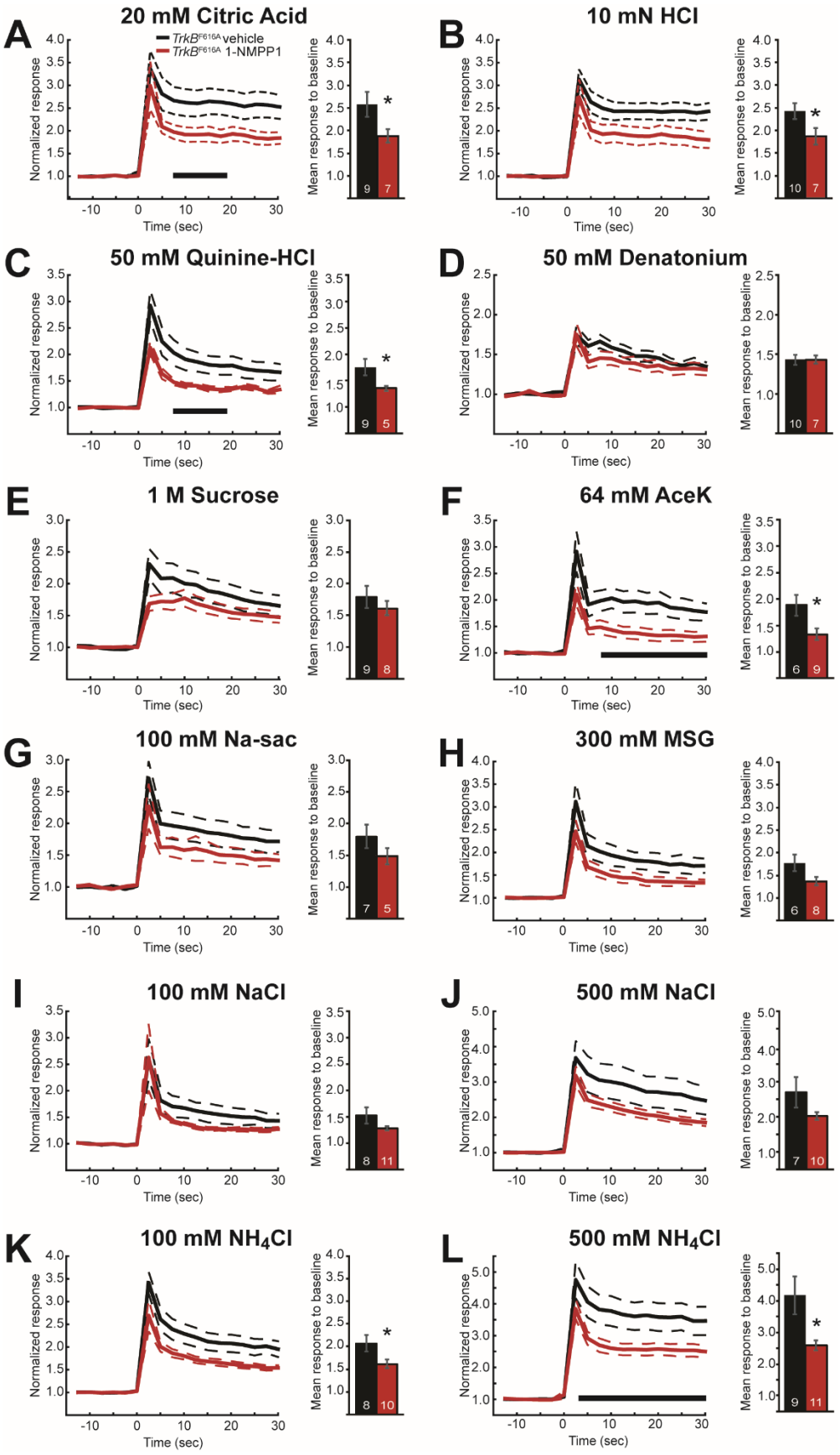
A
20 mM Citric Acid
TrkB^F616A vehicle
TrkB^F616A 1-NMPP1
B
10 mN HCl
C
50 mM Quinine-HCl
D
50 mM Denatonium
E
1 M Sucrose
F
64 mM AceK
G
100 mM Na-sac
H
300 mM MSG
I
100 mM NaCl
J
500 mM NaCl
K
100 mM NH4Cl
L
500 mM NH4Cl
Normalized response
Time (sec)
Mean response to baseline
*

Figure 3.5. **Baseline normalized responses were disrupted by blocking TrkB-signaling**. Peri-stimulus time histograms represent the average normalized responses for *TrkB*^{F616A} mice that received vehicle (black) and 1-NMPP1 (red) to sour stimuli: A,B) Citric Acid and HCl, to bitter stimuli: C,D) Quinine-HCl and Denatonium; to sweet stimuli: E-G) Sucrose, Acesulfame K^+ (AceK) and Na-saccharin (Na-sac); to umami stimuli: H) MSG; to salts: I-L) salts: NaCl and NH_4Cl. Data shown as mean (solid line) ± standard error of the mean (SEM) (dashed lines) over a 45s window. Bin size = 2.5 seconds. Bar graphs represent the average 20s of the evoked normalized response. Figure legend applies to A-L. *p<0.05. Black bar represents 3 or more consecutive bins that were significantly different over time.

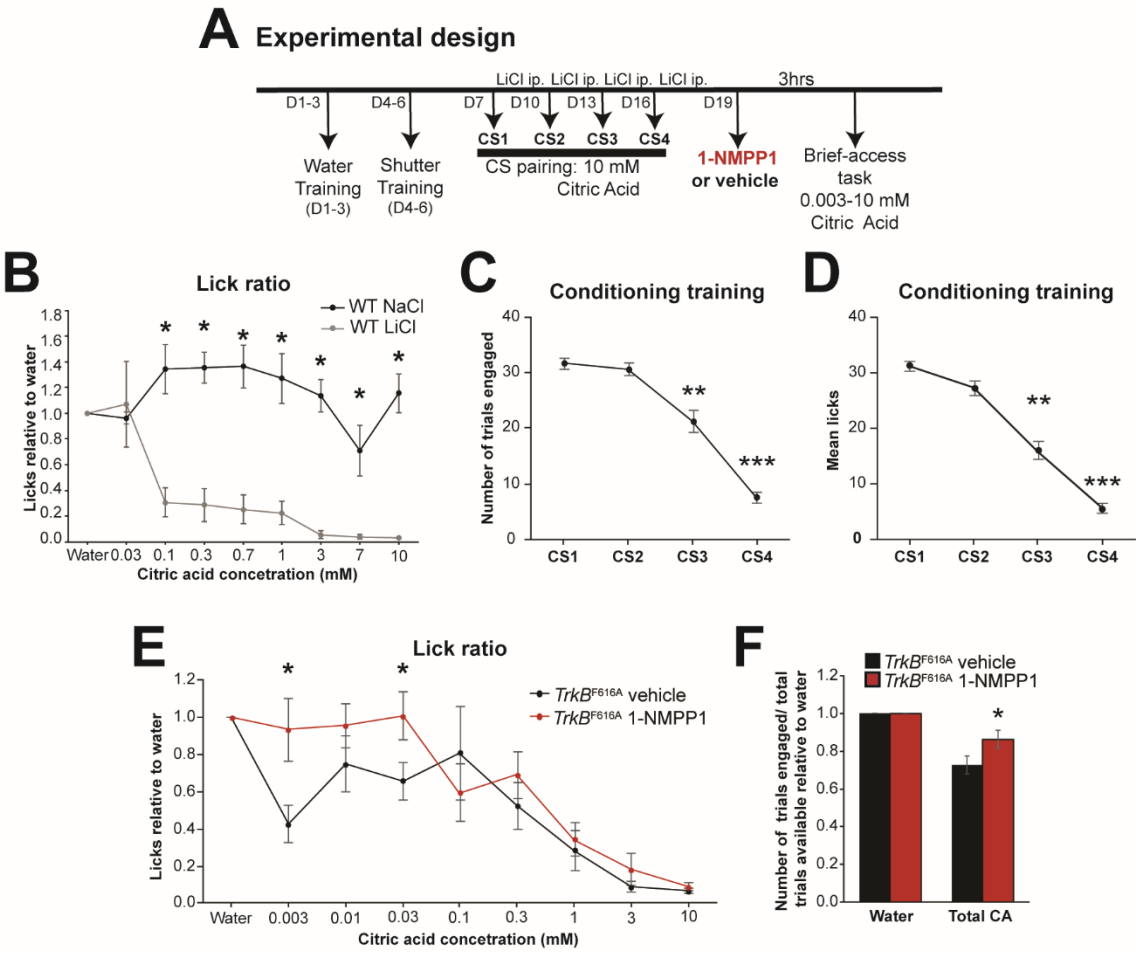

Figure 3.6. ***TrkB*^{F616A} mice injected with 1-NMPP1 did not detect lower concentrations of citric acid on a brief-access task after conditioned taste aversion**. A) Experimental design for a brief-access task combined with conditioned taste aversion (CTA). Wild type mice were conditioned by paring 10 mM citric acid (CS) with LiCl injections. The brief-access task consisted of randomized presentations of water and 8 different concentrations of citric acid stimuli, with a maximum time of 15 minutes. Each trial consisted of a maximum of 10s with additional 5s after first lick followed by a 7.5s intertrial interval. B) Conditioning LiCl injections paired with 10mM citric acid (CS) significantly

reduced the number of licks to 10 mM citric acid and lower concentrations in wild type mice by the third and fourth CS pairing. C) *TrkB*^{F616A} mice were trained as described in A. Conditioning training significantly reduced the number of trials engaged and D) mean licks to 10 mM citric acid by the third and fourth day of training. On test day, *TrkB*^{F616A} mice received 1-NMPP1 or vehicle for 3 hours prior to the behavioral assay. E) *TrkB*^{F616A} mice with vehicle had significantly fewer licks to 0.003 mM and 0.3 mM of citric acid compared to water (*p<0.05) than *TrkB*^{F616A} mice treated with 1-NMPP1. F) The number of trials engaged per citric acid concertation were not significantly different between *TrkB*^{F616A} mice that received vehicle compared to 1-NMPP1 group. These data indicate that blocking TrkB signaling prevents the reduction in lick rate to lower concentrations of citric acid, which would normally occur following conditioned taste aversion to 10mM citric acid. Data shown as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01. ***p<0.01.

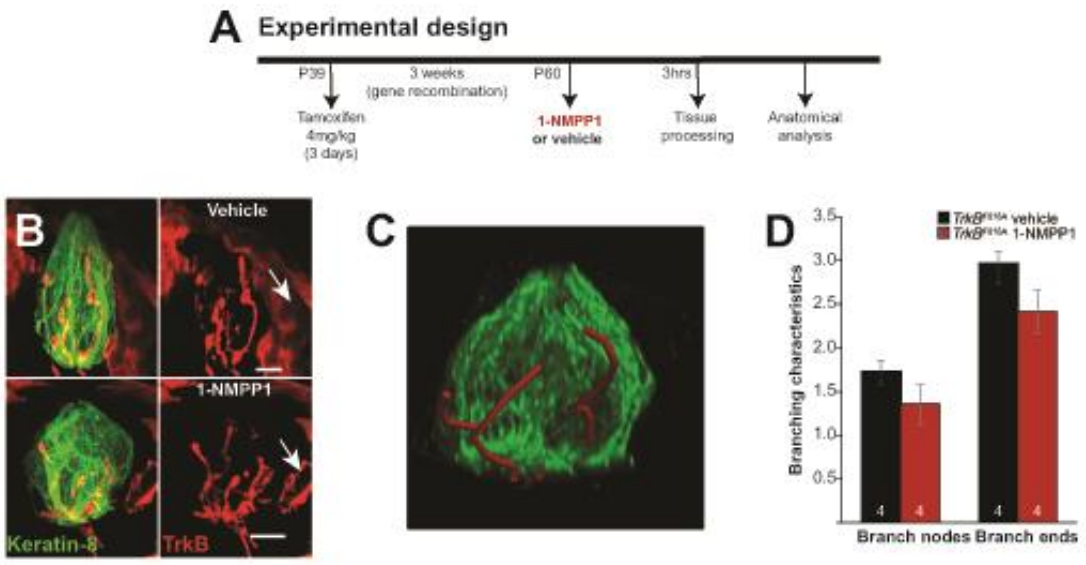

Figure 3.7**. 1-NMPP1 had no effect on branching characteristics in TrkB afferent fibers.** A) Experimental design for anatomical analysis of TrkB fibers innervating taste buds. *TrkB*$^{CreER/F616A}$::tdTomato mice received low doses of tamoxifen. B) Representative image of taste buds from sections of *TrkB*$^{CreER/F616A}$::tdTomato mouse tongues, after mice were treated with vehicle (top) or 1-NMPP1 (bottom). After 3 weeks, gene recombination labels tdTomato TrkB-expressing fibers (red) within the taste buds (green). TrkB-expressing epithelial cells were also labeled (arrows). C) Representative trace of TrkB fibers (red) within a taste buds (green). D) The number of branch nodes and branch ends were not significantly for each individual labeled fiber branches entering a taste bud between *TrkB*$^{Cre\ ER/F616A}$::tdTomato mice injected with vehicle (black) and 1-NMPP1 (red). Data shown as mean ± standard error of the mean (SEM). Scale bar = 10µm.

Table 1. Schedule of behavioral paradigm used for wild type mice.

Day	Stage	Time	Solution and duration	Time	Solution and duration
Day 1-3	Training	AM	Stationary spout (15 mins)	PM	Water (1.5 mins)
Day 4-6	Training	AM	Moving spout (15mins)	PM	Water (1.5 mins)
Day 7	Conditioning	AM	CS (1): 10mM citric acid (15mins) + LiCl (ip) or NaCl (ip)	PM	Water (1.5 mins)
Day 8-9	Recovery	AM	Water (15 mins)	PM	Water (1.5 mins)
Day 10	Conditioning	AM	CS (2): 10mM citric acid (15mins) + LiCl (ip)) or NaCl (ip)	PM	Water (1.5 mins)
Day 11-12	Recovery	AM	Water (15 mins)	PM	Water (1.5 mins)
Day 13	Conditioning	AM	CS (3): 10mM citric acid (15mins) + LiCl (ip) or NaCl (ip)	PM	Water (1.5 mins)
Day 14-15	Recovery	AM	Water (15 mins)	PM	Water (1.5 mins)
Day 16	Conditioning	AM	CS (4): 10mM citric acid (15mins) + LiCl (ip) or NaCl (ip)	PM	Water (1.5 mins)
Day 17-18	Recovery	AM	Water (15 mins)	PM	Water (1.5 mins)
Day 19	Testing	AM	0.03-10 mM citric acid and water (15 mins)		

3.4. Discussion:

I investigated whether TrkB signaling influences taste function by disrupting activation of the TrkB receptor using a genetic-chemical approach, which blocks TrkB signaling with 1-NMPP1 (Chen et al., 2005; Johnson et al., 2008; Y. Liu et al., 2012; Sompol et al., 2011; Vandenberg et al., 2015; Wang et al., 2009). Visual inspection of the raw data suggested that blocking TrkB-signaling reduced responses to NH_4Cl . Therefore, I normalized each 2.5s of each trial to the average of baseline. Reporting responses proportional to baseline has been used previously to examine taste function with experimental modifications in which NH_4Cl responses were also altered (Larson et al., 2015; Vandenbeuch et al., 2013). However, this is the first study that used peri-stimulus time histograms (PSTH) to compare temporal changes in a group of taste responses from whole nerve recordings (ex. TrkB groups: vehicle vs 1-NMPP1). I found that four different stimuli were reduced for at least 10s (7.5s-17.5s post-stimulus). These stimuli cut across functional taste modalities; however, it could be argued that sour stimuli were the most impacted because it was the only modality where multiple stimuli were reduced. Consistent with our electrophysiological data, I found that blocking TrkB signaling reduced sensitivity to low concentrations of citric acid solutions in a brief-access test. Lastly, these functional changes did not result from morphological changes of TrkB fibers innervating taste buds.

Multiple different mechanisms could explain how BDNF-TrkB signaling could directly modulate taste function. In the CNS, BDNF release is activity

dependent (Greenberg, Xu, Lu, & Hempstead, 2009; Park & Poo, 2013; Tao, West, Chen, Corfas, & Greenberg, 2002; Wong et al., 2015), and although activity-dependent release of BDNF has not been verified in the taste system, it is certainly possible. In the CNS, direct application of BDNF evokes neuronal excitability within seconds (Black, Smith, McIsaac, & Ferguson, 2018; Kafitz et al., 1999; McIsaac & Ferguson, 2017). A large number of both presynaptic and post-synaptic mechanisms have been identified that could mediate this effect (Blum & Konnerth, 2005; Chao, 2003; Greenberg et al., 2009; Park & Poo, 2013).

One possible mechanism in which BDNF modulates taste function is by binding to the full-length TrkB receptor on the presynaptic cell to influence neurotransmitter release (Greenberg et al., 2009; Park & Poo, 2013; Tao et al., 2002; Wong et al., 2015). Consistent with a potential pre-synaptic role, phosphorylated TrkB is expressed in taste receptor cells (I. V. Nosrat et al., 2012). BDNF regulation of neurotransmitter release has been best studied for glutamate (Carvalho, Caldeira, Santos, & Duarte, 2008; Kafitz et al., 1999; Z. Zhang, Fan, Ren, Zhou, & Yin, 2013), which may function and a neuromodulator in the taste bud (Vandenbeuch & Kinnamon, 2016). However, BDNF-TrkB-signaling could also interact with serotonin which is an established neurotransmitter for taste (Y. J. Huang et al., 2005; Larson et al., 2015; Roper, 2006). Throughout the CNS, BDNF appears to be expressed in neurons that also express serotonin (Martinowich & Lu, 2008; Pietrelli et al., 2018). In addition, although the mechanisms are largely unclear, BDNF signaling and serotonin function synergistically, such that BDNF reduction exacerbates the behavioral

effects in serotonin transporter knockouts (Ren-Patterson et al., 2005). Depression studies have suggested that BDNF may influence serotonin reuptake (Martinowich & Lu, 2008; Ren-Patterson et al., 2005). While serotonin, released from type III cells activate geniculate neurons via $5HT_{3a}$ (Y. J. Huang et al., 2005; Larson et al., 2015), it is unclear whether TrkB signaling regulates taste responses specifically in $5HT_{3a}$-containing neurons. However, studies have shown that selective serotonin receptor inhibitors (SSRIs) altered sensitivity for bitter and sweet in depressed patients (Heath, Melichar, Nutt, & Donaldson, 2006).

In addition to modulating neurotransmitter function, BDNF binding to the TrkB receptor can directly activate membrane channels such as Nav1.2 (Ahn et al., 2007), Nav1.9 (Blum et al., 2002), TRPC3 (Amaral & Pozzo-Miller, 2007a; H. S. Li et al., 1999; Rose et al., 2004), and Kiv1.3 (Tucker & Fadool, 2002), which play roles in membrane excitability. Interestingly, Nav1.9 is a non-voltage gated sodium channel capable of modulating function of CNS neurons. The most important and best studied role of Nav1.9 is in the function of small unmyelinated sensory neurons that mediate pain (Hoffmann et al., 2017; Kanellopoulos & Matsuyama, 2016). Nav1.9 channels could play a similar role in gustatory neurons as they are also mostly small unmyelinated neurons (Yokota & Bradley, 2016). Recent studies have shown that geniculate neurons that express full-length TrkB receptor and project to the oral cavity (Phox2b+) express Nav1.9 (98%) and also Kiv1.3 (30%) to a much lower extent (Dvoryanchikov et al., 2017).

Because I injected 1-NMPP1 three hours before recordings, mechanisms that alter gene/protein expression must also be considered. BDNF has been shown to result in transcriptional changes within 1 hour of stimulation (Gokce et al., 2009; X. Zhang & Poo, 2002). BDNF can regulate both P2X3 and Calcitonin gene-related peptide (CGRP) expression (Qiao & Grider, 2007; Simonetti et al., 2008). Taste receptor cells release ATP which binds P2X3/P2X2 receptors in nerve fibers and is required for taste function (Finger et al., 2005; Ishida et al., 2009; Kinnamon & Finger, 2013; Roper, 2006; Staikopoulos et al., 2007; Vandenbeuch et al., 2015). In trigeminal neurons, P2X3 receptors are up-regulated by BDNF (Simonetti et al., 2008), so it is possible that P2X3 receptors are normally maintained by BDNF such that they are reduced when TrkB-signaling is blocked. Sensory neurons in the DRG, upregulate CGRP expression in response to BDNF stimulation (Qiao & Grider, 2007). Consistent with CGRP regulation as a possible mechanism, CGRP is expressed in ~50% of the TrkB+ geniculate ganglion neurons that project to the oral cavity (Dvoryanchikov et al., 2017). If TrkB-signaling regulates CGRP expression in taste neurons, increased CGRP release could (A. Y. Huang & Wu, 2015) provide positive feedback of neurotransmitter release from taste receptor cells (A. Y. Huang & Wu, 2015).

In the adult taste system, most Car4-positive (94%) cells (Type III) transduce sour stimuli (Chandrashekar et al., 2009) and form synapses with afferent fibers (C. L. Yee et al., 2003) and express BDNF (T. Huang et al., 2015). For this reason, I hypothesized that in adulthood, TrkB expressing fibers would connect with this cell type and blocking TrkB-signaling would primarily impact

sour taste responses. Consistent with this idea, behavioral and electrophysiological responses to citric acid and HCl were suppressed and the behavioral response to citric acid was disrupted. However, I also found that other taste stimuli were reduced including quinine-HCl (bitter), acesulfame K^+ (sweet) and high concentrations of NH_4Cl (salt). There are several possible explanations for the results. First, although this population of Car4-expressing cells is required for sour taste (Chandrashekar et al., 2009), many of these cells also respond to other taste stimuli (Tomchik, Berg, Kim, Chaudhari, & Roper, 2007; Yoshida et al., 2009). Second, a small percentage (11%) of Type II cells, PLCβ2 cells, which transduce bitter, sweet and umami stimuli, also express BDNF in adulthood (Tang et al., 2017). Therefore, TrkB-expressing fibers may also innervate these taste receptor cell types. TrkB is expressed in 50% of the TrkB neuron population (Chapter II) which is a substantial portion of the taste neuron population and perhaps some of these fibers innervate non-BDNF expressing taste receptor cells. Lastly, given the large number of possible ways in which BDNF-TrkB signaling can influence function, multiple mechanisms could be involved, only some of which directly influence communication between BDNF-expressing taste receptor cells and TrkB nerve fibers.

I found that short-term blockage of TrkB signaling in adulthood reduced evoked taste responses to multiple taste stimuli. In a recent study, when BDNF was removed from taste cells over a ten-week period relative responses to NaCl, sucrose, quinine and citric acid were unaffected (C. Sun et al., 2018). There are several possible explanations for these differences. First, TrkB is capable of

binding more than one ligand (Chao, 2003; E. J. Huang & Reichardt, 2003), and it is possible that either NT3 or NT4 compensate for the loss of BDNF (Chao, 2003; E. J. Huang & Reichardt, 2003). Second, BDNF can have both excitatory and inhibitory effects, which could cancel each other out (Eide et al., 1996; Fujita et al., 2011; Levine et al., 1995). Lastly, removal of BDNF may impact taste function similarly to blocking TrkB signaling, but reduction in NH_4Cl responses may have masked changes in relative taste responses (C. Sun et al., 2018). This seems particularly likely since chorda tympani fields were enlarged as a result of BDNF removal from the periphery (C. Sun et al., 2018). Consistent with previous studies, reduction of functional activity in the periphery promotes increased chorda tympani terminal field sizes in the NTS (Mangold & Hill, 2007; Skyberg et al., 2017; C. Sun et al., 2017).

The peripheral taste system is one of the most plastic sensory systems in the body. Since taste receptors cells die and are replaced, fibers must form connections with new cells. A large number of developmental molecules continue to be expressed in the adult taste system (Castillo-Azofeifa et al., 2017; Gaillard & Barlow, 2011; Gaillard et al., 2017; T. Huang et al., 2015; Meng et al., 2015; Miura & Barlow, 2010; Tang et al., 2017; C. Yee, Bartel, & Finger, 2005; C. L. Yee et al., 2003). Some of these molecules regulate the taste receptor cells, while others likely regulate nerve fiber growth, branching and connectivity in the taste bud (Lee et al., 2017; Meng et al., 2015; Tang et al., 2017). However, many of these molecules, including ephrins and semaphorins, can modulate nerve function independent of changes in fiber morphology or connectivity (Bi, Yue,

Zhou, & Plummer, 2011; Blum & Konnerth, 2005; Rose et al., 2004; Sahay et al., 2005). In this study I demonstrate that TrkB-signaling influences functional taste responses independent of changes in morphology. Therefore, it is critical to separate these potential roles with careful experimental design.

CHAPTER IV

GENERAL SUMMARY AND DISCUSSION

The main goals of this dissertation were 1) to define taste neurons based on TrkB expression and dependence during development and adulthood and 2) examine the functional role of TrkB signaling in taste. In chapter II, I examined when and how TrkB downregulation occurs in the geniculate ganglion. I found that TrkB expression and dependence divides taste neurons into subpopulations. I suggest that BDNF expression initially guides TrkB+ fibers to innervate taste buds during a critical developmental period (L. Ma et al., 2009). During this time, all taste neurons express and depend on TrkB, while the 9% of oral cavity-projecting neurons that are TrkB-independent are oral somatosensory. After this critical period, TrkB expression in Phox2b+neurons is downregulated, dividing these neurons into a TrkB+ and TrkB- subpopulations. In adulthood, BDNF likely maintains taste bud innervation of the TrkB+ but not the TrkB- taste fibers (Tang et al., 2017). In the adult taste system, these TrkB+ taste neurons likely play a different functional role than TrkB- neurons. Regardless, Phox2b+ neurons can be divided based on TrkB expression into three populations that innervate the oral cavity: a population that depends on TrkB during development and expresses TrkB in adulthood (50%), a taste population that depends on TrkB during development but down-regulates TrkB

by adulthood (41%) and finally, a likely oral somatosensory population that never expresses or depends on TrkB (9%).

Next, I sought to determine the role of TrkB-signaling in taste function, specifically, in the 50% adult taste neurons that continues to express TrkB receptor. In chapter III, I found that TrkB-signaling influences taste responses especially from neurons that form synapses and respond to sour stimulation. My data suggest that TrkB-signaling influences taste function in whole nerve recording and behavior. These functional changes were not due to changes in neuron morphology. I suggest that BDNF released from taste cells in an activity-dependent manner can modulate the release of other neurotransmitters important for communication between taste cells and nerve fibers such as serotonin and ATP (Finger et al., 2005; Y. A. Huang et al., 2011; Kinnamon & Finger, 2013; Larson et al., 2015). Based on my findings, BDNF-TrkB signaling might also facilitate transmission of taste information post-synaptically by opening channels that maintain the resting membrane potential and/or regulate neuron excitability (Ahn et al., 2007; Blum et al., 2002; Tucker & Fadool, 2002).

In future studies, it would be interesting to examine whether BDNF is released from taste cells in an activity dependent manner. One possibility could be to record from dissociated taste cells and measure with a BDNF-calcium indicator, BDNF levels before and after taste stimulation (Larson et al., 2015). Another possibility could be to measure mRNA BDNF levels transcribed from activity dependent exons (ex. exon III and IV) (Tao et al., 2002). Another study of main interest would be to record dissociated geniculate neurons from *TrkB*^{F616A}

mice and examine how their membrane properties change with applications of ATP, serotonin, CGRP, and other neurotransmitters before and after 1-NMPP1 (block TrkB-signaling), ANA-12 (TrkB antagonist) and/or SFK-PLCγ pathway inhibitor. Lastly, I would like to examine taste function in mice with TrkB-signaling blocked for more than 3 hours (24 hrs, 1 week, 2 weeks, and 4 weeks) to determine if the functional effects are consistent for sour responses or would affect across taste modalities since branching of TrkB neurons might be affected in long-term treatment (2 weeks or more).

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CURRICULUM VITAE

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Education:

Institution	**Degree** ***Department, Specialization***	**Date**
University of Louisville	Ph.D. *Anatomical Sciences and Neurobiology*	2015-2018
University of Louisville	M.S. *Anatomical Sciences and Neurobiology*	2012-2015
University of Kentucky	M.S. *Physiology*, *Medical Sciences*	2010
University of Puerto Rico	B.S. *Biology*	2007

Professional Memberships and Activities

2015-2018 Member of Society for Neuroscience
2014-2018 Member of Association for Chemoreception Sciences
2012-2018 Member of Louisville Society for Neuroscience Chapter
2009 Member of Society for Neuroscience
2005 Member of Puerto Rico Society of Microbiologists
2004-2007 Member of American Medical Student Association

Honors/Awards

Excellence in Neuroscience Research. Graduate Student Poster Competition, 3rd Place. Neuroscience Day, SFN Louisville Chapter. Louisville, KY 2017.

AChemS Travel Fellowships for Diversity**.** *Association for Chemoreception Sciences,* 2016

Teaching Experience

Courses:

2017 Developmental Neurobiology University of Louisville
Guest Instructor- 1 lecture
Organization of the Nucleus of the Solitary tract

2014 Developmental Neurobiology University of Louisville
Guest Instructor- 1 lecture
Organization of the Nucleus of the Solitary tract

2013 Medical Neuroscience University of Louisville
Teaching Assistant: Neuroanatomy laboratory
Medical and graduate students

2007 Biological Sciences University of Puerto Rico
Teaching Assistant and Biology Tutor
Undergraduate students

Community Involvement:

2014-17 Brain Awareness Week Kentucky Science Center
Volunteer for histology and comparative anatomy areas that have included activities for kids age 5-18

2017 Louisville Science Pathway (LSP) University of Louisville
Invited speaker
Title: TrkB signaling in taste development and development (June 2017)

2017 STEMinar: University of Louisville
Invited speaker
Title: TrkB signaling in taste development
(April 2017)

2016-17 Dupont Manual Regional Science Fair University of Louisville
Served as a jugde

Abstracts and Presentations

Abstracts from National/International Meetings:

Rios-Pilier J., A. Victoria Clements, Lundy R., Samuelsen C., Krimm R. F., Blocking BDNF-TrkB signaling reduces taste function. Neuroscience Day, SFN Louisville Chapter. Louisville, KY 2018.

Rios-Pilier J., Krimm R. F., Expression of the TrkB Receptor is Downregulated before Birth Dividing Taste Neurons into Three Subpopulations. Neuroscience Day, SFN Louisville Chapter. Louisville, KY. 2017.

Rios-Pilier J., A. Victoria Clements, Lundy R., Krimm R. F., Blocking BDNF-TrkB signaling reduces taste function, particularly, to sour stimuli. Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2017. Online.

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De Jesús-Burgos MI, Vázquez-Fuentes BM, Torres-Llenza V, **Ríos-Pilier J,** Comenencia-Ortiz EJ, Rodríguez S, Quiñones K, Pérez-Acevedo NL. (2006) Modulation of risk assessment behaviors through group I metabotropic glutamate receptors in the basolateral amygdala in estrogen-treated rats. IBNS.

Publications:

Articles Published in Peer-Reviewed Journals

Rios-Pilier J, Krimm RF. TrkB expression and dependence divides gustatory neurons into three subpopulations. Neural Development. 2018. Submitted.

Rios-Pilier J., A. Victoria Clements, Lundy R., Samuelsen C., Krimm R. F., Blocking BDNF-TrkB signaling reduces taste function. J Neuroscience. 2018. In preparation.

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NIH (minority fellowship) subaward to parent grant entitled: *Neurotrophin regulation of taste system development.*
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Principal Investigator: Robin F. Krimm, Ph.D.
Agency: National Institute of Deafness and Communicative Disorders
Period: 8/1/14-5/30/16

NIH (F31) Award entitled: *TrkB-expression divides taste neurons into subtypes*
(Summited 8/12/15 - Impact Score: 20)
Agency: National Institute of Deafness and Communicative Disorders
Period: 6/1/16-5/30/18