

8-2013

The role of neurotrophin receptors in taste development.

Da Fei

University of Louisville

Follow this and additional works at: <https://ir.library.louisville.edu/etd>

Recommended Citation

Fei, Da, "The role of neurotrophin receptors in taste development." (2013). *Electronic Theses and Dissertations*. Paper 430.
<https://doi.org/10.18297/etd/430>

This Doctoral Dissertation is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.

THE ROLE OF NEUROTROPHIN RECEPTORS IN TASTE DEVELOPMENT

By

Da Fei

B.A., University of Science and Technology of China, 2004

A Dissertation Submitted to the Faculty of the School of Medicine of the University of

Louisville in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Anatomical Sciences and Neurobiology

University of Louisville, School of Medicine

Louisville, Kentucky

August 2013

THE ROLE OF NEUROTROPHIN RECEPTORS IN TASTE DEVELOPMENT

By

Da Fei

B.A., University of Science and Technology of China, 2004

A Dissertation Approved on

May 28, 2013

By the following Dissertation Committee:

Robin F. Krimm, Ph.D. (Director)

Michal Hetman, Ph.D.

Robert F. Lundy, Jr., Ph.D.

J. Patrick Moore, Ph.D.

Jeffrey C. Petruska, Ph.D.

ACKNOWLEDGEMENTS

I would like to thank all the people that had helped and supported me during my Ph.D. study. Without them, it would not be possible for me to complete my dissertation research. I enjoyed being a student at University of Louisville, particular the Department of Anatomical Sciences and Neurobiology. The help and inspiration I received from people around me are invaluable and I greatly appreciate that. I am particularly grateful for the tremendous mentoring I have received from my advisor, Dr. Robin Krimm. I would like to thank her for her continuous guiding and support during my studies in the past five years. She is always available and ready to help, especially when I experienced the difficult moments during my research. Without her support, I could not make to this point. I would like to thank all the current and past members of the Krimm lab. They are the best colleagues to work with. They supported me by discussing my research projects, helping me in maintaining mice lines and so much more. I would like to thank my committee members, Drs. Michal Hetman, Robert Lundy, Patrick Moore, and Jeffrey Petruska for their support and constructive suggestions. Last, I would like to thank my family for their support and love.

ABSTRACT

THE ROLE OF NEUROTROPHIN RECEPTORS IN TASTE DEVELOPMENT

Da Fei

May 28, 2013

Brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) are two neurotrophins that play distinct roles in geniculate (taste) neuron survival, taste innervation and taste bud formation. These two neurotrophins activate the same receptors tyrosine kinase B (TrkB) and a pan-neurotrophin receptor (p75). While the roles of these neurotrophins have been well studied, it remains unclear how much BDNF and NT-4 function through TrkB and p75 to regulate taste development *in vivo*. In chapter 2, I compared taste development in *TrkB*^{-/-} mice and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice to determine if these deficits were similar. If so, this would indicate that the functions of both BDNF and NT-4 can be accounted for by TrkB signaling. I found that *TrkB*^{-/-} mice and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice lose the same number of geniculate neurons by E13.5, indicating that BDNF and NT-4 primarily function through TrkB to regulate geniculate neuron survival. Surprisingly, the few geniculate neurons remaining in *TrkB*^{-/-} mice are more successful in innervating the tongue and taste buds than those remaining in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. As a result these remaining neurons in the *TrkB*^{-/-} mice innervate and support the development of a surprising number of taste buds. In addition, these remaining neurons do not express the

TrkB receptor, indicating the either BDNF or NT-4 must function through an additional receptor to influence taste innervation and/or targeting.

The p75 receptor can function as either a pro-survival or pro-death factor during peripheral nervous system development. However, the role of p75 in taste development is unknown. In chapter 3, I examined neuron survival, taste bud formation and taste innervation in the $p75^{-/-}$ and $TrkB^{-/-}/p75^{-/-}$ mice. I found that at E13.5, the age after BDNF and NT-4 dependence is established, $p75^{-/-}$ mice did not lose geniculate neurons compared to the wild type mice. This finding indicates that p75 does not mediate neurotrophin neuronal survival at or before this age. Hybrid $TrkB^{-/-}/p75^{-/-}$ mice had the same number of geniculate neurons as $TrkB^{-/-}$ mice, suggesting that p75 does not induce neuron death in the absence of Trk-signaling like it does for some other systems. By E14.5, there is a loss of geniculate neurons in $p75^{-/-}$ mice which continues until E18.5. Also at this age, the pattern of chorda tympani nerve innervation was disrupted in the $p75^{-/-}$ mice. Specifically, the nerves avoid innervating the mid-region of the tongue. Due to the loss of innervation, the taste bud number was decreased in the $p75^{-/-}$ mice. It is possible that the neuron loss is due to this disrupted innervation pattern. Interestingly, $TrkB^{-/-}/p75^{-/-}$ mice have more taste buds than $p75^{-/-}$ mice. These additional taste buds are not innervated which suggests that p75 may function as a pro-death factor in the taste bud during development. Taken together, my results suggest that p75 does not mediate the neurotrophin survival function for taste neuron development. Instead, it is important for taste innervation and branching to the tongue middle line and could influence taste cell survival in the absence of innervation and/or neurotrophin signaling.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
CHAPTER 1	1
1.1 Adult taste system anatomy and function	1
1.1.1 Taste and taste buds	1
1.1.2 Taste bud innervation and taste signal transduction	2
1.1.3 Taste cells	3
1.1.4 Taste receptors	3
1.2 Neurotrophins in sensory neuron development.....	4
1.2.1 Neurotrophins	4
1.2.2 Role of neurotrophins in sensory neuron development	5
1.2.3 Neurotrophin receptors	5
1.3 Neurotrophins in taste development	7
1.3.1 Introduction.....	7
1.3.2 The development of geniculate ganglion.....	7
1.3.3 Taste nerve outgrowth and guidance	10
1.3.4 Taste papillae formation and innervation	11

1.3.5 Targeting of taste nerves to fungiform papillae	12
1.3.6 Taste bud induction: innervated or un-innervated ?.....	13
1.3.7 Taste bud differentiation, number and size.....	14
CHAPTER 2	16
2.1 Introduction.....	16
2.2 Materials and methods	18
2.2.1 Animals.....	18
2.2.2 Quantification of geniculate ganglion neuron number	18
2.2.3 Quantification of fungiform papilla number using scanning electron microscope (SEM)	20
2.2.4 Quantification of taste bud number.....	20
2.2.5 Quantification of the amount of innervation within a taste bud.....	21
2.2.6 Geniculate ganglia labeling using DiI.....	22
2.2.7 Quantification of innervation to the lingual epithelium.....	23
2.2.8 Quantification of TrkB-GFP expression in the geniculate ganglion.....	24
2.2.9 Data analysis.....	24
2.3 Results.....	25
2.3.1 BDNF and NT-4 both function primarily through TrkB to support geniculate ganglion neuron survival during embryonic development	25
2.3.2 <i>TrkB</i> ^{-/-} mice lose the same number of fungiform papillae but have more taste buds than <i>Bdnf</i> ^{-/-} / <i>Ntf4</i> ^{-/-} mice by birth	27

2.3.3 Fungiform taste buds were better innervated in <i>TrkB</i> ^{-/-} mice than <i>Bdnf</i> ^{-/-} / <i>Ntf4</i> ^{-/-} mice at P0	29
2.3.4 More taste fibers reach their targets in <i>TrkB</i> ^{-/-} mice than <i>Bdnf</i> ^{-/-} / <i>Ntf4</i> ^{-/-} mice during development.....	30
2.3.5 The remaining geniculate neurons do not express TrkB.....	32
2.4 Discussion	33
CHAPTER 3	40
3.1 Introduction.....	40
3.2 Methods.....	43
3.2.1 Animals.....	43
3.2.2 Quantification of geniculate ganglion neuron number	44
3.2.3 Quantification of taste bud number and taste bud innervations.....	45
3.2.4 Geniculate ganglia labeling using DiI.....	47
3.2.5 Data analysis	47
3.3 Results.....	48
3.3.1 The p75 receptor does not mediate neurotrophin survival/death during early development.....	48
3.3.2 Geniculate neurons were lost in <i>p75</i> ^{-/-} mice from E14.5 to E18.5	49
3.3.3 Taste nerves lose taste innervation to the mid-region of tongue in <i>p75</i> ^{-/-} mice.....	50
3.3.4 Taste buds are lost and taste bud innervation is reduced at P0 in <i>p75</i> ^{-/-} mice	51
3.4 Discussion	53

CHAPTER 4.....	57
FIGURES.....	63
REFERENCES	87
CURRICULUM VITAE.....	100

CHAPTER 1

GENERAL INTRODUCTION

1.1 Adult taste system anatomy and function

1.1.1 Taste and taste buds

Taste (gustation), is one of the five basic senses in vertebrates, among the others are vision, audition, olfaction and somatosensation. Through taste sensation, we are able to detect pleasure or harmful substances in foods and make wise decisions for our eating behaviors. Up to now, five essential taste senses have been characterized: sweet, bitter, sour, salty and umami (amino acid). In general, sweet, umami and low concentration of salty are considered as palatable tastes, which allow us to be aware of nutritionally rich food; while bitter, sour and high concentration of salty are unpalatable, which are considered to function in letting us avoid harmful molecules.

Taste in vertebrates is detected by peripheral receptors called taste buds. All vertebrates (except hagfish) have taste buds. In humans, taste buds are located on the surfaces of tongue, soft palate, pharynx, epiglottis, and upper esophagus. On the tongue, taste buds are housed in specialized epithelium placodes called papillae. Three types of papillae are found on human tongue (Figure 1): (1) Mushroom shaped fungiform papillae that scatter throughout the front area of the tongue. (2) Short vertical folds-like foliate

papillae at the lateral margins of the tongue. (3) Dome-shaped circumvallate papillae situated at the very back of the tongue.

1.1.2 Taste bud innervation and taste signal transduction

Taste buds that reside in different types of papillae are innervated by distinct nerves from the cranial ganglions (Figure 2). Fungiform papillae and part of the foliate papillae are innervated by chorda tympani nerve, and taste information received from these papillae is relayed to taste neurons in the geniculate ganglion. In addition, the geniculate ganglion innervates taste buds on the soft palate via the Greater Superficial Petrosal (GSP) nerve. Lastly, the geniculate ganglion also provides somatosensory innervation to the outer ear.

The circumvallate papillae and most of the foliate papillae are innervated by glossopharyngeal nerve from the petrosal ganglion. Both the geniculate and petrosal ganglia relay taste signal to a higher brain structure called nucleus of the solitary tract (NST). From there, taste information is transmitted through the parabrachial nucleus (in rodents) or directly to the ventral posteromedial nucleus of the thalamus (in primates). From the thalamus, taste information is relayed into the primary gustatory cortex in the insula.

1.1.3 Taste cells

In mammals, taste buds contain about 50-100 elongated epithelia cells and a few proliferative basal cells. Morphological studies have characterized four types of taste cells (Figure 3): dark cells, light cells, intermediate cells and basal cells ([1], [2], [3], [4]). Dark cells, which are the most numerous, are so called because they tend to accumulate basophilic dyes. Light cells, are so named due to their aversion for basophilic dyes. Intermediate cells, in some cases, may be undifferentiated cells which derive into dark and light cells ([5]). However, these three types of taste cells may represent different cell lineages ([1], [2], [3], [6]). Last, basal cells are small round cells at the base of taste buds. They are thought to be stem cells from which other cells are derived, in addition, they may function as interneurons in some vertebrates ([1], [2], [3]).

1.1.4 Taste receptors

Taste buds contain receptors for taste stimuli. Taste receptors for umami, sweet, bitter, salty and sour have been characterized (Figure 4). Umami and sweet are mediated by three G-protein-coupled receptors (GPCRs), including T1R1, T1R2 and T1R3. T1R1+3 function as a mammalian umami receptor and T1R2+3 as a sweet receptor ([7], [8], [9], [10]). Bitter is mediated by a family of highly divergent GPCRs (the T2Rs). Studies from mutants of *T2R* genes validated that T2Rs are essential and sufficient for bitter coding ([11], [12]).

Sour and salty have been shown as direct entry of H⁺ or Na⁺ through specialized membrane proteins. For sour, a member of TRP family, PDK2L1, has been identified

([13], [14], [15]). However, a large group of receptors have also been proposed to function in sour transduction ([16], [17], [18], [19], [20], [21], [22]). For salty, it has been shown that the epithelial sodium channel (ENaC) is responsible for Na⁺ taste receptor cell activation, however, it is likely only one of multiple mechanisms for Na⁺ taste transduction. ([21], [23]).

1.2 Neurotrophins in sensory neuron development

1.2.1 Neurotrophins

Neurotrophins are a group of structurally related trophic factors that are important for the survival, development, and function of the nervous system in vertebrates. Four members of the neurotrophic factor family have been characterized in mammals, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4).

The initial role of neurotrophins was described by the following central idea: innervated targets create limiting amount of growth factors that would balance the size of targets and the number of innervating neurons. NGF was the first neurotrophin that was purified, and it has been shown to support the survival of sympathetic and sensory spinal neurons ([24]). After the identification of NGF, other neurotrophins were purified and cloned. *In vitro* studies using explants of peripheral sensory organs had suggested that each neurotrophin functions in different (although overlapping) sensory neuron populations ([25]).

1.2.2 Role of neurotrophins in sensory neuron development

Sensory neurons that relay distinct modalities of sensory information tend to be segregated into different ganglia, thus sensory neurons in specific ganglia are often affected by different neurotrophins. However, for some ganglia, such as DRG and trigeminal sensory ganglia, neurons conveying different modalities of sensory information are mixed in the same ganglia, so differential neurotrophins regulate the neurons with different sensory modalities in these ganglia. *In vivo* studies with neurotrophin mutant mice have allowed the identification of different neurotrophin dependences for specific sensory ganglia. Table 1 shows the summary of neuronal loss in the sensory system of neurotrophin- and neurotrophin receptor- deficient mice.

In addition to the role in promoting neuronal survival, neurotrophins are also involved in multiple aspects of the development of the sensory system, including neuronal differentiation, axon elongation and target innervation, and synapse formation ([26]). In the taste system, most studies have been focused on the role of BDNF and NT-4. These two neurotrophins regulate taste neuron survival, taste nerve outgrowth and targeting and taste bud development.

1.2.3 Neurotrophin receptors

The neurotrophins function as homo-dimers, and activate protein tyrosine kinase receptors, which include three members: TrkA, TrkB, and TrkC. TrkA is the receptor for NGF, TrkB is the receptor for both BDNF and NT-4, and TrkC is the primary receptor for NT-3. NT-3 also activates TrkA and TrkB with less efficiency. The Trk receptor

family has a specific extracellular domain which is enriched in leucine and cysteine repeats, a consensus intracellular tyrosine kinase domain with a small interruption, and a short cytoplasm tail.

The activation of Trk receptors and downstream signaling pathways appears to promote survival in most neuronal populations ([26], [27], [28]). In addition to their survival role, the activation of Trk receptors also appears to regulate multiple aspects of nervous system development, including neural differentiation, axon outgrowth and target innervation, and synapse formation etc.

In addition to the full length Trk receptor, truncated isoforms of Trk receptors have been characterized. For example, the mammalian *trkB* locus undergoes alternative splicing to produce a full length TrkB receptor and a truncated receptor, which lacks the intracellular tyrosine kinase domain. This truncated TrkB receptor may function in a negative way by inhibiting neuronal survival by interaction with the full length TrkB receptor ([29]).

Each of these neurotrophins also binds to the low-affinity neurotrophin receptor p75, a trans-membrane glycoprotein which belongs to the tumor necrosis factor receptor superfamily ([30], [31]). The p75 receptor contains four negatively charged cysteine-rich extracellular repeats, and a conserved cytoplasmic domain. The activation of p75 leads to multiple outcomes. Especially, depending on the existence of Trk receptors, p75 can function as either a pro-survival or a pro-death factor ([32]).

1.3 Neurotrophins in taste development

1.3.1 Introduction

The function of the taste system depends on precise connections between taste neurons and peripheral targets. These connections are made during embryonic and postnatal development. Numerous molecular factors contribute to the development of the peripheral taste system. For the purpose of this introduction, I will focus primarily on those molecules (especially neurotrophins) involved in the development of the taste system including: the development of neurons in the geniculate ganglion, the outgrowth of taste nerves, nerve growth into peripheral targets, and the development of the taste papillae and taste buds.

1.3.2 The development of geniculate ganglion

The geniculate ganglion is derived from epibranchial placodes although some of the glia are derived from neural crest ([33], [34], [35]). Signals from the pharyngeal endoderm and a series of transcription factors are important for the formation of epibranchial placodes ([36], [37], [38], [39], [40]). For example, BMP-7, a member from the bone morphogenetic protein family, is vital for pharyngeal endoderm to induce placodal neurons ([37]). Neurogenin2, a basic-helix-loop-helix (bHLH) protein, is important for the neuroblasts within placodes that give rise to geniculate ganglion ([38]). In addition, the home domain transcription factors, Phox2a and Phox2b are also essential for the differentiation of the geniculate ganglion ([38], [39], [40]).

During geniculate ganglion genesis, the number of neurons within the geniculate ganglion is determined by the balance between the number of neural precursors born in the ganglion, the number that differentiate into neurons, and the number that undergoing apoptosis. Typically, it is believed that neurons are overproduced and then undergo a programmed cell death during development. In the geniculate ganglion, neuron production peaks around E12 in rats ([41]), which roughly equals E10 in mice (Table 2). The peak of neuronal cell death is around E14.5 in mice. It is the age that taste nerves first penetrate the fungiform papillae epithelium to form neural buds ([42],[43], [44]). This finding that the peak of geniculate neuron death is during target innervation is in line with the idea that factors derived from the fungiform papillae mediate neuron survival.

BDNF and NT-4 are important regulators for geniculate neuron number. *Bdnf*^{-/-} and *Ntf4*^{-/-} mice lose about half of their geniculate neurons during development. Hybrid *Bdnf*^{-/-}/*Ntf4*^{-/-} mice lose around 90% of their geniculate neurons ([45], [46], [47], [48]). In addition, in the mice that overexpress BDNF or NT-4, geniculate neuron numbers are increased ([49], [50]). These findings indicate that there are multiple subpopulations in the geniculate ganglion that differ in their neurotrophin dependence. There are at least two possibilities for these subpopulations. One possibility is that there are two subpopulations, one subpopulation depends on BDNF, and the other subpopulation depends on NT-4. This would account for the findings of the neuron number losses in those mutant mice. Another possibility is that there are also two subpopulations, but one subpopulation depends on both BDNF and NT-4, another subpopulation depends on either BDNF or NT-4. In this scenario, in *Bdnf*^{-/-} and *Ntf4*^{-/-} mice, the first subpopulation of neurons would be lost, but the second subpopulation remains due to the existence of

either NT-4 or BDNF. However, in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, both the first and the second subpopulation would be lost. These possibilities are not mutually exclusive. If both possibilities are true this indicates that there may be four types of geniculate ganglion neurons regarding their neurotrophin dependence: BDNF-dependent, NT-4-dependent, BDNF- or NT-4-dependent, and BDNF- and NT-4-dependent. It is unclear how these different neurotrophin dependencies will be sorted out. In addition, these possibilities are controversial to the pro-survival role of NT-3. *Ntf3*^{-/-} mice lose about 47% of their geniculate neurons ([47]), so it is certain that some overlap must exist in the geniculate neuron dependence.

The timing of geniculate neuron loss in *Bdnf*^{-/-} and *Ntf4*^{-/-} mice is different. Geniculate neurons were lost earlier in development in *Ntf4*^{-/-} mice (starts around E11.5), indicating an early role of NT-4 in geniculate ganglion development ([51]). Interestingly, a second set of neuron loss occurs in *Ntf4*^{-/-} mice during E14.5 to E16.5, suggesting NT-4 mediates geniculate neuron survival at two different time points; one before target innervation and one after target innervation. After E16.5, geniculate neuron number starts to increase in *Ntf4*^{-/-} mice. In *Bdnf*^{-/-} mice, geniculate neurons are lost starting from E13.5, which is just before or at the onset of target innervation, and the loss continues to E18.5, which is after target innervation ([52]). Neuronal loss in *Bdnf*^{-/-} mice was prevented by removal of *Bax*, indicating BDNF mediates geniculate neuron number by preventing cell death rather than promoting cell proliferation ([52]). However, in *Ntf4*^{-/-} mice, activated caspase-3, which is increased in BDNF mutants, was unaffected ([51]). Together, these findings suggest that NT-4 regulates neuron survival at different stages than BDNF and BDNF mediates neuron survival for a longer embryonic period. These findings also

indicate that instead of a simple switch in neurotrophin dependence from NT-4 earlier and BDNF later, each neurotrophin has multiple roles and utilizes multiple mechanisms for mediating geniculate neuron development.

1.3.3 Taste nerve outgrowth and guidance

During development, chorda tympani nerves follow a precise, spatially restricted pathway to the tongue surface ([53]), which must be controlled by a series of molecular cues from the adjacent environment. *In vitro* studies have shown that geniculate axon growth requires the adding of BDNF and NT-4, but not NT-3 to the culture media ([54]). In addition, NT-4 is more capable of stimulating and attracting neurite extension than BDNF ([55]). However, mutants of BDNF or NT-4 do not affect the ability of chorda tympani nerves to reach the tongue ([56]). These results indicate that although BDNF and NT-4 are important for taste nerve outgrowth, they seem to function redundantly *in vivo* to support the outgrowth of axons.

A variety of attractive and repulsive guidance cues have been validated for axon outgrowth and guidance, including: semaphorins, ephrins, netrins and Slits ([57]). Ephrins, semaphorins and Slits act as repellents, but netrins can be either attractants or repellents ([58], [59], [60], [61]). However, in the taste system, although any of these guidance molecules may regulate axon guidance of geniculate neurons, most of them remain un-investigated except semaphorin 3A (Sema3A). Sema3A is expressed in the tongue and required for both trigeminal and geniculate axon guidance ([62], [54], [63]). Sema3A expression decreases from the medial to the lateral tongue surface. This specific

expression pattern prevents premature and aberrant growth of trigeminal and geniculate axons into the mid-region of the tongue. In addition, *Sema3A* functions as a repellent factor preventing premature penetration of the epithelium when geniculate axons reach the epithelium surface. *Sema3F*, another member of semaphorins, is also expressed by lingual epithelium and maybe also involved in controlling geniculate axon guidance ([63]).

In addition, neurotrophins have been shown to interact with *Sema3A* and *Sema3F* to mediate geniculate axons outgrowth and guidance ([63], [64]). For example, NT-4, but not BDNF, facilitates the repellent effects of *Sema3A* and *Sema3F* ([63]). In *Ntf4* overexpressing mice, chorda tympani fibers remain underneath the epithelium, as if repelled by the lingual epithelium ([65]). On the other hand, NGF has been shown to reduce sensitivity of somatosensory neurons to *Sema3A* ([64], [66]). BDNF may have a similar role in reduce geniculate axon sensitivity to *Sema3A in vivo*, although it has not been shown *in vivo* ([63]).

1.3.4 Taste papillae formation and innervation

Fungiform papillae are present at the tongue as early as E14.5 ([42]). Signaling factors involved in epithelial patterning such as sonic hedgehog (*Shh*), the bone morphogenic proteins (*Bmp*), *Noggin*, fibroblast growth factor 8 (*FGF 8*) and *Wnt* ligands, have also been shown to orchestrate papillae formation ([67], [68], [69], [70]).

The initial formation of fungiform papillae occurs without innervation; however, the maintenance of papillae requires innervation ([71]). Specifically, *in vitro* cultured E14

tongues which lack intact sensory innervation lose fungiform papillae after 6 days in culture. These results suggest that initial formation of fungiform papillae is nerve-independent, while the maintenance of papillae is nerve-dependent.

1.3.5 Targeting of taste nerves to fungiform papillae

Fungiform papillae are arranged in a specialized pattern on the tongue surface ([72]). The chorda tympani provide innervation to these fungiform papillae. At E14.5, chorda tympani nerves reach the tongue epithelium, penetrate the epithelium and form a distinctive nerve ending called neural bud ([42]). BDNF regulates the targeting of chorda tympani nerves ([73], [74], [75], [54], [76], [50], [56]). Specifically, BDNF is expressed in fungiform papillae before they are innervated ([73], [74], [75], and has been shown to attract chorda tympani nerves outgrowth *in vitro* ([54]). In addition, BDNF overexpression (BDNF-OE) in mice disrupted the normal innervation pattern, and chorda tympani nerves failed to innervate most fungiform papillae ([50]). Instead, non-taste filiform papillae were innervated in BDNF-OE mice, indicating that BDNF expressed in non-taste papillae attracted the chorda tympani nerves and became innervated ([65]). Furthermore, in the *Bdnf*^{-/-} mice, chorda tympani nerves failed to innervate fungiform papillae, instead, branching was increased and many non-taste papillae were innervated. Lastly, targeting was selectively disrupted when BDNF was conditionally removed from the epithelia ([56]). Taken together, these results indicate that BDNF is required for targeting during a critical period of development.

1.3.6 Taste bud induction: innervated or un-innervated ?

Unlike the fungiform papilla, whether or not taste bud formation is nerve-independent is controversial. The long-standing view is that neural innervation is required for the induction of taste buds ([77], [44], [78]). According to this model, the initial induction of fungiform taste buds occurs when the chorda tympani nerves invade the mesenchyme of the tongue and innervate the fungiform papillae.

Alternatively, recent studies support the nerve-independent view ([79], [80], [81]). For example, taste buds can develop in a grafted pharyngeal region that was taken from a donor axolotl embryo, prior to its innervation by neural elements ([82]). It is clear that in axolotl taste buds do not require innervation to develop. However, taste buds development in the axolotl may be different from those in mammals ([81]).

A study of *TrkB*^{-/-} mice also implied that taste buds could be induced without innervation ([83]). In *TrkB*^{-/-} mice, most of the geniculate neurons were lost, and so it was assumed that the taste nerves to the tongue were also lost. Surprisingly, a significant number of taste buds developed in *TrkB*^{-/-} mice. The authors concluded that this finding supported the work in axolotl and that taste buds do not require innervation for their development. However, this study lacked direct evidence examining the nerve fibers innervating the tongue and remaining taste buds. Also, the number and size of remaining taste buds was not specified. My experiments in Chapter 2 directly overcome the shortcomings of this earlier study and provide very different conclusions.

1.3.7 Taste bud differentiation, number and size

Following initial induction, taste bud differentiation occurs. The source of factors controlling taste bud differentiation is under debate. Since epithelial signals contribute to taste bud induction, it is also possible that factors from the epithelium contribute to the taste bud differentiation. However, it is equally feasible that taste bud differentiation is under control of molecules derived from nerve fibers. In addition, these are not mutually exclusive ideas. Taste buds are complicated sensory organs with an ever increasing number of cell subtypes, which may require numerous factors from multiple sources for their differentiation. Additional experimental evidence is needed to determine which mechanisms regulate the differentiation of taste buds.

In addition to not knowing the factors that regulate taste bud differentiation, the question of what factors control the final number and size of adult taste buds also remains unclear. Taste bud size and number could be regulated by various cellular and molecular factors in the lingual and extra-lingual taste bud populations ([84]). Among those factors, neurotrophins have been shown regulate taste bud number during development. Fungiform papillae and taste buds were lost in both *Bdnf*^{-/-} and *Ntf4*^{-/-} mice ([85], [86], [87], [88]). Compared to wild type mice, *Bdnf*^{-/-} mice showed a loss of 59% in the total number of developing taste buds from the tongue, however, *Ntf4*^{-/-} mice only showed 18% reduction in the total number taste buds. Tongues from hybrid of *Bdnf*^{-/-} and *Ntf4*^{-/-} mice only had a few taste buds remaining. In addition, taste bud size is impacted in neurotrophin knockout mice. There was a 59% decrease in the volume of taste buds in the tongues of *Bdnf*^{-/-} mice, and an 80% decrease in the volume of taste buds in the tongues of hybrid of *Bdnf*^{-/-} and *Ntf4*^{-/-} mice. There was no significant change of taste bud size in

the tongues of *Ntf4*^{-/-} mice ([89]). In addition, more taste papillae and taste buds were lost postnatally in *Ntf4*^{-/-} mice compared to wild type mice ([85]), and taste buds in *Ntf4*^{-/-} mice do not contain taste pores, indicating a role of NT-4 in the maintenance of fungiform papillae and taste buds. Furthermore, a recent study in transgenic mice in which BDNF overexpression was driven by an α -gustducin promoter (Gust-BDNF), taste buds are significantly larger and have more taste cells, taste innervation is also markedly increased, which could be due to direct influence of BDNF on taste cell number of in adult mice ([90]). These evidences together indicate BDNF has a different role than NT-4 in regulating taste bud development.

Taken together, BDNF and NT-4 have been shown expressed differently and function differentially to regulate geniculate neuron survival, axon growth and targeting, and taste bud development. Interestingly, BDNF and NT-4 mediate these different aspects of taste development by binding the same receptors, the tyrosine kinase receptor, TrkB, and the pan-neurotrophic receptor, p75. How BDNF and NT-4 function uniquely through the same receptors to regulate taste development is unclear. The first step in addressing this goal is to determine the role of each of these receptors in taste system development. I addressed this issue by performing the following studies to determine if and when TrkB and/or p75 signaling is required for taste neuron survival, taste axon growth to the tongue and targeting, and taste bud development.

CHAPTER 2

GENICULATE GANGLION NEURONS REMAINING IN THE *TRKB*^{-/-} MICE INNERVATE AND SUPPORT THE DEVELOPMENT OF TASTE BUDS

2.1 Introduction

During development, taste neurons, whose cell bodies are located in the geniculate ganglia, innervate specific regions of gustatory epithelium with a precise number of neurons. The development of geniculate ganglia and the formation of peripheral connections of taste neurons are highly regulated by two neurotrophins brain-derived neurotrophic factor (BDNF) and Neurotrophin-4 (NT-4). Specifically, *Bdnf*^{-/-} mice and *Ntf4*^{-/-} mice each lose about 50% of geniculate neurons by birth ([45], [46]). NT-4 regulates the survival of geniculate neurons earlier than BDNF via a different mechanism ([51]). In addition to regulating geniculate neuron number, BDNF in the tongue epithelium is also important for gustatory neuron targeting ([56], [91]). As a result of the targeting deficit, *Bdnf*^{-/-} mice have a more severe loss of innervation to taste buds than *Ntf4*^{-/-} mice ([87], [92], [86], [89])

BDNF and NT-4 function via the same receptors, TrkB and p75 ([93], [27]). Mice lacking TrkB lose more nodose-petrosal ganglion neurons than BDNF or NT-4 mutants

alone ([45]), indicating that both of these neurotrophins may function via TrkB to influence neuron number during development. Similarly, both hybrid *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice have been shown to lose a significant number of geniculate neurons ([46], [83]). Taste buds and fungiform papillae have been shown to require innervation for their maintenance by birth, and lack of innervation has resulted in taste bud loss in both *Bdnf*^{-/-} and *Ntf4*^{-/-} mice by birth ([87], [50]). Surprisingly, *TrkB*^{-/-} mice do develop fungiform papillae and taste buds ([83]) while a substantial loss of fungiform papillae has been observed by birth in hybrid *Bdnf*^{-/-}/*Ntf4*^{-/-} mice ([94]). It is unclear why the effects in hybrid *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice should be so different in the developing taste system. One explanation is that these two studies did not use similar quantification methods; therefore, the results are difficult to compare. Therefore, it still remains unclear how much BDNF and NT-4 function occurs through TrkB to regulate geniculate neuron survival and taste bud innervation during development. It is also unclear why taste buds develop in *TrkB*^{-/-} mice in spite of loss of most of taste neurons.

To address these questions, we directly compared the development of the gustatory system in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice. These data provided direct evidence that BDNF and NT-4 primarily function through TrkB to regulate taste neuronal survival in vivo. However, a small subpopulation of these geniculate neurons does not require or express TrkB, and these neurons can innervate and support the development of a surprisingly large number of taste buds.

2.2 Materials and methods

2.2.1 Animals

Heterozygous *TrkB*^{+/-} (stock no. 002544), *Bdnf*^{+/-} (stock no. 002266), *Ntf4*^{+/-} (stock no. 002497) mice were acquired from Jackson Laboratories (Bar Harbor, Maine, USA,). *TrkB*^{tauEGFP} mice were a generous gift from Dr. Ginty's lab ([95]). *TrkB*^{-/-} embryos were obtained by breeding heterozygous mice with a target mutation of the *TrkB* gene. *TrkB*^{tauEGFP/-} embryos were obtained by breeding *TrkB*^{tauEGFP} mice with *TrkB*^{+/-} mice. *Bdnf*^{-/-}/*Ntf4*^{-/-} mice were obtained by breeding *Bdnf*^{+/-}/*Ntf4*^{-/-} mice. Animals were genotyped using polymerase chain reaction. Embryonic mice were obtained from time breeding of females that were examined for plugs the following morning. The day a plug was positively identified was designated embryonic day 0.5 (E0.5). Animals were cared for and used in accordance with the guidelines of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

2.2.2 Quantification of geniculate ganglion neuron number

Embryos aged E11.5 (*TrkB*^{-/-} n=3, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=3), E12.5 (*TrkB*^{-/-} n=3, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=3) and E13.5 (*TrkB*^{-/-} n=3, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=3) were transcardially perfused with ice cold 4% phosphate-buffered paraformaldehyde (PFA). Following perfusion, embryos were post-fixed overnight in 4% PFA. Following fixation, embryo heads were dissected and moved to 70%

ethanol and processed for paraffin embedding. Genuiculate ganglion neurons were visualized by class III β -tubulin (TUJ-1) antibody as previously described ([52]). Briefly, serial sections (5 μ m) of paraffin embedded embryos were collected on SuperFrost Plus slides (Fisher Scientific). Paraffin was removed by immersion in Citrisolv overnight. Following rehydration and endogenous peroxidase blocking, slides were treated for antigen retrieval in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, dH₂O; pH 6). Sections were washed in PBS and blocked for 1 hr in blocking solution (PBS, 5% goat serum, 0.25% Triton X-100), and were incubated overnight in mouse anti- β -III tubulin antibody (1:500, Covance, Princeton, NJ, USA; catalog #MMS-435P) in blocking solution. On the following day, sections were washed and incubated for 1.5 h in biotinylated anti-mouse secondary antibody (1:250, Vector Laboratories, Burlingame, CA, USA; #BA-2000) in blocking solution, and visualized with an ABC diaminobenzidine reaction kit (Vector Laboratories, Burlingame, CA, USA; #PK-6200).

For genuiculate ganglion volume measurement, the size of genuiculate ganglion in each section was measured and multiplied by the section thickness (5 μ m) to derive the volume of the single section; these volumes were added to derive the total volume for the entire ganglion. For the measurement of genuiculate neuron number, the TUJ-1 antibody was used to identify and count neuronal profiles in sections where the nucleus was visible. Neuronal profiles were counted in six representative sections per ganglion. The volumes of genuiculate ganglion in these six sections were also measured. The total number of neuronal profiles of the entire ganglion was estimated as the product of the number of profiles per volume of the counted section \times the total volume of the entire ganglion. The total number of neurons per ganglion was estimated by multiplying the number of total

neuron profiles by a correction factor to compensate for the presence of a nucleus in multiple sections (Abercrombie, 1946). The correction factor was calculated according to the formula: $N = n \times [T/(T \times D)]$, where N is the estimated total number of neurons, n is the number of nuclear profiles, T is the measured section thickness, and D is the average diameter of the nuclei ([52]).

2.2.3 Quantification of fungiform papilla number using scanning electron microscope (SEM)

Mice at day of birth (*TrkB*^{-/-} n=3, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=5) were anesthetized and transcardially perfused with ice cold 4% PFA. Tongues were dissected and post-fixed in 1% aqueous OsO₄ for 2.0 –2.5 h, washed in buffer, and successively dehydrated in a graded series of ethanol and then hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate from the tongues in a desiccator overnight. Tongues were mounted onto stubs, sputter-coated with gold and examined by SEM (Phillips 505). Digital SEM images were captured at 130x magnification to distinguish fungiform from filiform papillae. Individual fungiform papillae were imaged at 1770x magnification.

2.2.4 Quantification of taste bud number

Mice at day of birth (*TrkB*^{-/-} n=5, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=5, and wild-type n=4) were anesthetized and transcardially perfused with ice cold 4% PFA. The front of the tongue containing the fungiform field was separated and post-fixed in 4% PFA for two hours. Tongues were then placed in 30% sucrose overnight as cryoprotectant. The following day,

tongues were embedded in OCT (Sakura Finetek USA, Inc., #4583). Serial sagittal sections of the tongue (25um) were collected onto SuperFrost Plus slides (Fisher). For antigen retrieval, sections were heat dried overnight, rehydrated, placed into citrate buffer (pH 6.0), heated for 15 min in a boiling water bath, and incubated for 10 min at RT. The slides were washed in PBS and incubated overnight in 1:50 rat anti-TROMA1 antibodies (Developmental Studies Hybridoma Bank) in PBS. The next day, the slides were rinsed in PBS (3×5 min), and incubated in anti-rat Alexa 555 secondary antibodies (1:500, Molecular Probes) for two hours. After washing in PBS (3×5 min), the slides were dehydrated, cleared in Citrisolv and cover-slipped using DPX mounting medium (Fluka). The sections were examined in order, and the taste buds were followed across sections so that each taste bud was only counted once.

2.2.5 Quantification of the amount of innervation within a taste bud

Mice from day of birth (*TrkB*^{-/-} n=4, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=4, and wild-type n=4) were anesthetized and transcardially perfused with ice cold 4% PFA. The front of the tongue containing the fungiform field was separated and post-fixed in 4% PFA for two hours. Tongues were then placed in 30% sucrose overnight as cryoprotectant. The following day, tongues were embedded in OCT (Sakura Finetek USA, Inc., #4583). Serial sagittal sections of the tongue (25um) were collected onto SuperFrost Plus slides (Fisher). For antigen retrieval, sections were heat dried overnight, rehydrated, placed into citrate buffer (pH 6.0), heated for 15 min in a boiling water bath, and incubated for 10 min at RT. For primary antibodies, in addition to rat anti-Troma1 antibody, rabbit anti-P2X3 antibody

(1:500, Millipore, #AB5895) was used to label taste fibers. Secondary anti-rat Alexa 488 (green) and anti-rabbit Alexa 555 (red) antibodies (1:500, Molecular Probes) were used to visualize taste buds and taste fiber respectively.

Confocal stacks of optical sections with a Z step of 0.5 were imaged of 3-5 taste buds from every mouse of each genotype (*TrkB*^{-/-} n=4, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=4, and wild-type n=4) and then analyzed by ImageJ (<http://rsbweb.nih.gov/ij/>). The area occupied by the taste bud in each image section was measured; areas were summed and multiplied by section thickness (0.5um) to calculate taste bud volume. The area occupied by P2X3 positive staining within the outlined taste bud was also measured in each optical section; these areas were summed and multiplied by section thickness (0.5µm) to measure volume of innervation within the taste bud. The percentage of the taste bud that was occupied by innervation was determined by dividing the volume of P2X3 label by the volume of the Troma1 label.

2.2.6 Geniculate ganglia labeling using DiI

Embryos at ages E14.5, E15.5, E16.5 and E18.5 were anesthetized and transcardially perfused in ice cold 4% (PFA). Tongues were post fixed in 4% PFA overnight. DiI labeling was performed as described previously ([50]). Embryos were incubated at 37°C for 2–8 weeks depending on the age of the embryo. The tongue was then dissected, examined, and photographed using a fluorescent dissecting microscope (Leica MZFL) equipped with a camera (QImaging CE). Images were collected from tongues of *TrkB*^{-/-}, *Bdnf*^{-/-}/*Ntf4*^{-/-}, and wild-type mice at the following ages: E14.5 (*TrkB*^{-/-}

n=4, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=3) , E15.5 (*TrkB*^{-/-} n=4, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=5) E16.5 (*TrkB*^{-/-} n=6, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=4, and wild-type n=3) E18.5 (*TrkB*^{-/-} n=4, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=5).

2.2.7 Quantification of innervation to the lingual epithelium

Embryos at E16.5 (*TrkB*^{-/-} n=3, *Bdnf*^{-/-}/*Ntf4*^{-/-} n=3, and wild-type n=3) were anesthetized and transcardially perfused with ice cold 4% PFA. The front area of the tongue containing the fungiform field was separated and post-fixed for another two hours. Tongues were then placed in 30% sucrose overnight, and embedded in OCT (Sakura Finetek USA, Inc., #4583) the following day. Serial sagittal sections of the tongue (25um) were collected onto SuperFrost Plus slides (Fisher). For antigen retrieval, sections were heat dried overnight, rehydrated, placed into citrate buffer (pH 6.0), heated for 15 min in a boiling water bath, and incubated for 10 min at RT. The slides were washed in PBS and incubated overnight in mouse anti-2H3 antibody (1:100, Developmental Studies Hybridoma Bank) and rabbit anti-P2X3 antibody (1:500, Millipore, #AB5895) in PBS. The next day, the slides were rinsed in PBS (3×5 min), and were incubated in anti-rabbit Alexa 488, anti-mice Alexa 555 secondary antibodies (1:500, Molecular Probes) for two hours. After washing in PBS (3×5 min), the slides were dehydrated, cleared in Citrisolv, and cover-slipped using DPX mounting medium (Fluka). The sections were examined in order, and each instance where the nerve fibers invaded the epithelium was quantified. Each of these locations was examined in serial sections so that each fiber bundle that invaded the epithelium was only counted once.

2.2.8 Quantification of TrkB-GFP expression in the geniculate ganglion

Embryos at E13.5 (*TrkB^{tauEGFP/-}* n=3, and wild-type n=3) were anesthetized and transcardially perfused in ice cold 4% PFA. The head was dissected and post-fixed overnight in 4% PFA. The following day, the head was cryoprotected in 30% sucrose overnight. The next day, the tissue was embedded in OCT (Sakura Finetek USA, Inc., #4583). The embryo heads were sectioned at 25um and collected onto SuperFrost Plus slides (Fisher). The slides allowed drying at 40 °C for 1 hr. The slides were rinsed 3x5 min in PBST (PBS with 2.5% triton), blocked in 5% normal serum in PBST for 1hr, and then incubated overnight at RT in chicken anti-GFP antibodies (1:1000, Invitrogen, #A11122) and rabbit anti-P2X3 antibodies (1:500, Millipore, #AB5895) in blocking solution. The next day, the slides were rinsed in PBST (3x5 min), and then incubated in anti-chicken Alexa 488, anti-rabbit Alexa 555 secondary antibodies (1:500, Molecular Probes) for 1 hour. After they were washed in PBST (4x5 min), the slides were mounted with fluoromount-G (SouthernBiotech, #0100-01). The number of GFP positive and/or P2X3 positive neurons were counted. The ratio of neurons expressing both TrkB and P2X3 versus the neurons expressing P2X3 was calculated.

2.2.9 Data analysis

The total neuron number and total volumes were compared between genotypes on embryonic days E11.5, E12.5 and E13.5 using a two-way analysis of variance (ANOVA). The fungiform papillae number and area, the taste bud number and volume, taste bud innervation data were compared using one-way analysis of variance (ANOVA). The

alpha levels were set at $p < 0.05$ for all statistical comparisons. The data were described as mean \pm S.E.M. in the text and figures.

2.3 Results

2.3.1 BDNF and NT-4 both function primarily through TrkB to support geniculate ganglion neuron survival during embryonic development

BDNF and NT-4 have been shown to regulate gustatory neuron development at different ages. *Ntf4*^{-/-} mice start to lose geniculate neurons at E11.5, while *Bdnf*^{-/-} mice do not lose geniculate neurons until E13.5 ([52], [51]), both *Bdnf*^{-/-} mice and *Ntf4*^{-/-} mice lose approximately half of their geniculate neurons by birth. To understand if these two neurotrophins regulate geniculate neuron loss via the same receptor TrkB, we quantified the geniculate neuron number in wild-type, *Bdnf*^{-/-}/*Ntf4*^{-/-}, and *TrkB*^{-/-} mice from E11.5 to E13.5. We reasoned that if geniculate neurons were lost with a similar degree and timing in *Bdnf*^{-/-}/*Ntf4*^{-/-} hybrid and *TrkB*^{-/-} mice, then TrkB likely mediates the full effects of these two ligands.

First we compared the volume of geniculate ganglion in wild-type, *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice. At E11.5, there were no differences in geniculate ganglion volume in wild-type ($8.4 \pm 0.88 \times 10^5 \text{ um}^3$), *Bdnf*^{-/-}/*Ntf4*^{-/-} ($6.7 \pm 0.35 \times 10^5 \text{ um}^3$) and *TrkB*^{-/-} mice ($7.6 \pm 0.85 \times 10^5 \text{ um}^3$) (Figure 5 A,B,C; Figure 6A). At E12.5, the volume of geniculate ganglion was reduced by 36% ($p < 0.01$) in *Bdnf*^{-/-}/*Ntf4*^{-/-} ($5.8 \pm 1.01 \times 10^5 \text{ um}^3$) and by 76% ($p < 0.001$) in *TrkB*^{-/-} mice ($2.2 \pm 0.08 \times 10^5 \text{ um}^3$) compared to wild-type mice (9.0 ± 1.54

$\times 10^5 \text{ um}^3$); also the volume of geniculate ganglion was significantly smaller in *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} ($p < 0.01$) (Figure 5D,E,F 6A). At E13.5, the volume of geniculate ganglion in wild-type mice increased ($p < 0.05$), while the volume of geniculate ganglion in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice continued to decrease ($p < 0.01$). There was no significant difference between the volumes of the geniculate ganglion in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice at this age. These results show that during the early developmental ages, while geniculate ganglion volume in the wild type mice is increasing, there is a significant reduction in the volume of geniculate ganglion in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice, however, the reduction is slightly delayed in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice compared to *TrkB*^{-/-} mice.

Next we quantified the number of neurons present in the geniculate ganglion. Geniculate neurons were easily identified because of their clear nucleus and dark cytoplasm when stained with a neuron-specific marker anti- β -III tubulin antibody (TUJ-1) (Figure 5G). Overall, there was a slight increase (17%; $p < 0.01$) in geniculate neuron number in wild-type mice from E11.5 to E13.5, while there was a continuous neuron loss in *Bdnf*^{-/-}/*Ntf4*^{-/-}, and *TrkB*^{-/-} littermates during the same embryonic period ($p < 0.01$; Figure 2B). Specifically, at E11.5, compared to wild-type mice (650 ± 23), geniculate neuron number in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (451 ± 16) was reduced by 31% ($p < 0.01$) while the neuron number in *TrkB*^{-/-} mice (355 ± 58) decreased 45% ($p < 0.01$, Figure 6B). There was 21% fewer neurons in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice ($p < 0.01$) at this age. This finding is consistent with earlier studies indicating that neuron number is a more sensitive measure of cell loss than geniculate volume at these early ages when neurons are small ([52]). By E12.5, there was 16% increase of geniculate neuron number in the wild-type mice

(756±68) compared to the E11.5 littermates ($p < 0.01$, Figure 6B). There was no significant change in neuron number in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice at E12.5 compared to E11.5. However, neuron number in *TrkB*^{-/-} mice (144±16) decreased 59% between ages E11.5 and E12.5 ($p < 0.01$). *Bdnf*^{-/-}/*Ntf4*^{-/-} mice have lost 48% of geniculate neurons compared to the wild-type mice ($p < 0.01$), which is similar to the neuron loss in *Ntf4*^{-/-} mice ([51]). Neuron number in the *TrkB*^{-/-} mice was reduced by 81% compared to the wild-type littermates ($p < 0.01$). There were 64% fewer neurons in *TrkB*^{-/-} mice than in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice on E12.5 ($p < 0.01$). One explanation for this surprising finding is that other neurotrophin members, such as NT-3, might also function through TrkB to regulate neuronal survival during these early ages. At E13.5, the neuron number was reduced by 80% in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (149±24; $p < 0.01$) and 87% in *TrkB*^{-/-} mice (97±18; $p < 0.01$) compared to the wild-type mice (763±41, Figure 6B). Thus, by E13.5, the bulk of the ganglion is gone in both genotypes. Furthermore, the possible function of NT-3 is short-lived, because by E13.5 there was no difference in geniculate neuron number between *Bdnf*^{-/-}/*Ntf4*^{-/-}, and *TrkB*^{-/-} mice. These data together suggested that there was a continuous neuron loss in both BDNF/NT-4 and TrkB mutants between E11.5 and E13.5. This loss was equivalent by E13.5, indicating BDNF and NT-4 primarily function through TrkB to regulate neuron survival during the early developmental ages.

2.3.2 *TrkB*^{-/-} mice lose the same number of fungiform papillae but have more taste buds than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice by birth

Minimal taste bud loss has been reported in *TrkB*^{-/-} mice in spite of the tremendous amount of geniculate neuron loss we observed ([83]). However, taste buds

were not quantified in this study. Therefore, we sought to re-examine this issue by quantifying both fungiform papilla and taste bud number in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice at day of birth. To determine if fungiform papillae were lost in mice lacking both BDNF and NT-4 or TrkB, tongues from *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice were processed for scanning electron microscopy (SEM) and the number and size of fungiform papillae were quantified (Figure 7). There were significant reduction in fungiform papillae number at P0 in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (61±5) and *TrkB*^{-/-} mice (53±2) compared to wild-type mice (84±2, p<0.01), but there was no significant difference in the loss between these two mutants. We also found that there was a reduction of 32% and 35% in the size of fungiform papillae in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (132±6 μm²) and *TrkB*^{-/-} mice (126±8 μm²) compared to wild type (195±12 μm²), respectively (Figure 3). Like the number of fungiform papillae, there was no difference between the sizes of fungiform papillae in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice.

Although fungiform papillae were lost in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice compared to wild type mice, we were surprised by how many fungiform papillae remained considering the severe neuron loss in these genotypes. However, many of the fungiform papillae that we examined could lack taste buds. To test this possibility, fungiform taste buds were examined from *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, *TrkB*^{-/-} mice and wild type mice at P0. Taste buds were visualized by using anti-Troma1 (Figure.8 A,B,C). As hypothesized fewer taste buds than fungiform papillae remained for both *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice (Figure 8 D). There was a substantial taste bud loss for both *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (8±1) and *TrkB*^{-/-} mice (31±3) compared to wild type mice (93±3, p<0.001). More interestingly, there were more taste buds remaining in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-}

^{-/-} mice ($p < 0.001$). To determine if remaining tastes were the same size in all genotypes we measured taste bud volume. We found that taste buds in both mutant mice were smaller than the wild-type mice ($p < 0.001$), however, there was no difference between the mutant genotypes (Figure. 8 E).

2.3.3 Fungiform taste buds were better innervated in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice at P0

Taste buds are thought to be supported by innervation ([77], [96], [97], [98]). Since more taste buds remained in the *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice at P0, it is possible that these taste buds are better innervated. To test this possibility, taste buds and taste nerves were visualized with anti-Troma1 and anti-P2X3, respectively. P2X3 has been shown to be a good marker of gustatory innervation ([99]). Taste buds that were innervated by P2X3 positive fibers (Figure 9 A,B,C) and those that were not innervated (Figure 9 E,F) were counted in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice (Figure 9 G). There were significantly more innervated taste buds in *TrkB*^{-/-} mice ($p < 0.05$) than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. There were no differences in the number of un-innervated taste buds in *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. These results suggest that the extra taste buds in *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} mice are innervated, which indicates that there might be more taste innervation to the tongue surface in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice.

We also noticed that innervated taste buds appeared to have more taste innervation in *TrkB*^{-/-} than in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. To measure the innervation, the volumes occupied by the taste bud (Troma1) and the taste nerve (P2X3) within the taste bud for

each optical image were taken in individual taste buds (Figure.9 D). In wild type mice, the taste bud volume was $1441 \pm 227.3 \text{ um}^3$, while the volume of P2X3 positive nerves were $678 \pm 146.7 \text{ um}^3$ indicating that roughly half the taste bud is occupied by innervation in wild type mice at birth (Figure 9 H). The proportion of the taste bud occupied by innervation was substantially reduced in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice ($p < 0.001$, Figure 9 H), where the taste bud volume was $313 \pm 37.7 \text{ um}^3$ while the volume of P2X3 positive nerves were $46 \pm 5.2 \text{ um}^3$. In *TrkB*^{-/-} mice, the taste bud volume was $321 \pm 35 \text{ um}^3$, the volume of P2X3 positive nerves were $129 \pm 9.2 \text{ um}^3$, so roughly 40 percent of the taste bud was occupied by innervation. This was not significantly different from the wild type mice but higher than the proportion of the taste bud that was innervated in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice ($p < 0.01$) (Figure. 9 H). Together these data suggest that the remaining taste buds in *TrkB*^{-/-} mice are better innervated than those in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice at birth.

2.3.4 More taste fibers reach their targets in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice during development

To determine if *TrkB*^{-/-} mice have more taste innervation to the tongue than the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, we labeled chorda tympani axons with the lipophilic tracer, DiI, to examine the innervation pattern in the tongue. At E14.5, in wild type mice, chorda tympani fibers branch from the base of the tongue toward the surface. Previously we have shown that chorda tympani fibers defasciculate and form a structure called a “neural bud” as they invade the epithelium (Figure 10 A [42], [56]). At E14.5, in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice (Figure 10 B,C), there were no visible fibers on the dorsal surface of the

tongue, which suggests that either there are too few chorda tympani axons remaining to innervate the tongue or the growth of the axons is delayed in the mutants.

To determine if the chorda tympani fibers in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice were delayed in reaching the tongue and if there were more fibers reaching the target in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, we examined the innervation patterns in the later ages E15.5, and E18.5. At the later ages, wild type mice and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice had very similar innervation patterns as on E14.5 (Figure 10 A, D, F). At E15.5 wild type fiber bundles branched from the base of the tongue toward the lingual surface and form a neural bud (Figure 10 H). In the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, at E15.5 fibers did not branch to the surface of the tongue, and in rare cases where innervation was observed at the epithelial surface, thin wispy branches, lacking neural buds were observed. These findings are similar to that which was observed previously for *Bdnf*^{-/-}/*Ntf4*^{-/-} mice ([56]). By the age of E18.5, there was virtually no innervation to the tongue of *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Images are not shown because there was little to photograph. However, in the *TrkB*^{-/-} mice, starting at E15.5, there was clear innervation into the tongue (Figure 10 E,G). The number of these branches is noticeably fewer than the wild type littermates, due to the dramatic loss of geniculate neurons. In most tongues at all ages from E15.5 to E18.5 a few chorda tympani fibers appeared to reach the tongue surface (Figure 10 I, J, K arrows). However, the fiber bundles were so thin in *TrkB*^{-/-} mice it was difficult to be certain if they invaded the epithelial surface.

To determine whether the additional innervation seen in the tongues of *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} invaded the tongue epithelium, we double-labeled tongues at E16.5 with anti-P2X3 (Green) and anti-Neurofilament (Red) at E16.5 (Figure

11). While fiber bundles invading the epithelium were substantially smaller in both *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice compared with wild type mice (Figure 11 B, C, compared to A), all three genotypes had some P2X3 positive nerve fibers that invaded the epithelium. The number of locations where fibers penetrated the epithelium was quantified in serial sections. Compared to the wild type mice (107±6), there were fewer locations of innervation within the tongues of the *TrkB*^{-/-} mice (21±3) and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (8±2). In spite of the obvious loss of innervation in both mutant genotypes, there were significantly more locations of innervation in *TrkB*^{-/-} mice than the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, demonstrating more gustatory innervation in the *TrkB*^{-/-} mice and this greater amount of taste innervation in *TrkB*^{-/-} mice than in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice accounts for the larger number of taste buds in these mice.

2.3.5 The remaining geniculate neurons do not express TrkB

It is unclear why gustatory neurons remain to provide greater innervation to the tongue in *TrkB*^{-/-} mice than the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Because these remaining neurons are better at innervating the tongue in *TrkB*^{-/-} mice than in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, the implication is that they can respond to either BDNF or NT-4. One possibility is that this innervation is being maintained by a truncated form of the TrkB receptor that is not deleted in the *TrkB*^{-/-} mice ([100]), but binds BDNF and/or NT-4. The TrkB receptor has a full-length signaling form, but also truncated forms that may or may not signal ([101], [29]). The *TrkB* mutant mice used in this study still express truncated forms of TrkB ([29]; [102]). It is possible that these remaining neurons express truncated TrkB, and in the absence of the full length TrkB receptor they are supported by the truncated form of this receptor. Alternatively, this subpopulation which remains in absence of the full length TrkB

receptor may lack all forms of TrkB and could be maintained by an alternate mechanism. To determine if the remaining neurons in *TrkB*^{-/-} mice express TrkB, *TrkB*^{tauEGFP/+} mice were bred with *TrkB*^{+/-} mice to get *TrkB*^{tauEGFP/-} littermates. In *TrkB*^{tauEGFP/+} mice, GFP is expressed in all cells that have either truncated or full length forms of the TrkB receptor ([95]). Since by the age of E13.5, *TrkB*^{-/-} mice have already lost most geniculate neurons, we chose this age to examine whether the remaining neurons in *TrkB*^{-/-} mice express TrkB. Geniculate neurons were double stained with anti-P2X3 (red) and anti-GFP (Green) (Figure 12). In the wild type mice, 94±1% of the P2X3 expressing geniculate neurons also expressed TrkB. In contrast, in the *TrkB*^{tauEGFP/-} mice, only 11±2% of the geniculate neurons expressed TrkB. This indicates most of the neurons that are retained in TrkB mutants are part of the 6% population that is normally negative for all forms of the TrkB receptor. Combined with previous results, this suggests there is a small population of TrkB-independent neurons in the geniculate ganglion that do not express TrkB, but still need BDNF and/or NT-4 to regulate their nerve innervation patterns and to support taste bud formation.

2.4 Discussion

In this study, we compared taste neuron survival, taste bud development and target innervation in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice to determine the extent to which BDNF and NT-4 function through TrkB in regulating taste development. Our data show that by age E13.5, *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice lose most geniculate ganglion neurons. This neuron loss is similar in degree between these two mutants. In addition,

most taste innervation to the tongue was lost in hybrid *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice. In addition, both mutant genotypes lose most of their taste buds. Taken together these findings indicate that BDNF and NT-4 function primarily through TrkB to regulate taste neuron survival.

Although much of the role of BDNF and NT-4 occurs through TrkB during development, subtle differences between the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and the *TrkB*^{-/-} mice provide surprising insight into the possible role of other neurotrophins may regulate the development of the taste system. One such finding was that the loss of neurons in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice was slightly delayed compared to the neuron loss in *TrkB*^{-/-} mice at E12.5, suggesting that other neurotrophins may function as survival factors temporally at this age. One possibility is neurotrophin-3 (NT-3), which has also been shown to function via TrkB to support sensory neuron survival [25]. It is reported that *Ntf3*^{-/-} mice lose between 25% and 47% of the geniculate ganglion neurons [103], [47], which strongly indicates that NT-3 is required for the geniculate ganglion neuron survival. It is well known that many neurons require more than one neurotrophin to survive. In particular, during early development neurons sometimes depend on multiple neurotrophins at various stages of development [104]. For example, many sensory neurons of the trigeminal ganglion require both BDNF and NT-3 early in development but then later become NGF dependent [105]. In fact another ganglion complex, the nodose/petrosal, which also contains taste neurons, is dependent on NT-3 before any other neurotrophins and this dependence also begins on day E12.5 [106]. Likewise, geniculate neurons may be influenced by multiple factors early in development such that NT-3 could function as a temporary survival factor when BDNF is absent. Consistent with this assertion is the

finding that although *Ntf3*^{-/-} mice show normal taste bud size and number [87], *Ntf3*^{-/-}/*Bdnf*^{-/-} double mutants show a much greater loss in taste bud number than *Bdnf*^{-/-} mice alone [107]. This finding suggests that NT-3 may have a more important role in supporting taste neurons and bud development when BDNF is also absent.

In addition, the difference in the geniculate neuron number of the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and the *TrkB*^{-/-} mice could be due to other factors that bind to TrkB receptor. For example, G protein-coupled receptor ligands (e.g. adenosine), which have been shown to trans-activate the Trk receptors in the absence of neurotrophins, may contribute to the different neuron number seen in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and the *TrkB*^{-/-} mice at E12.5. *In vitro* data indicates that adenosine and adenosine agonists can activate Trk receptor phosphorylation, which via a mechanism that requires adenosine 2A receptor ([108], [109]). *In vivo* studies indicate that motor neuron survival after facial nerve injury is significantly enhanced by the activation of TrkB receptor by adenosine agonists ([110]). Adenosine has also been shown to enhance sweet taste through adenosine receptor, indicating its role involving the taste system ([111], [112]). Adenosine receptors are expressed in the sympathetic neurons as well as the autonomic ganglia ([113]), indicating they may also be expressed in the geniculate ganglion. It is possible that adenosine trans-activate TrkB receptor in the absence of BDNF and/or NT-4 and promotes geniculate neuron survival during early development. Taken together, these findings indicate that other factors such as adenosine can also function through TrkB to support geniculate neuron survival during early developmental ages, however, these roles are temporary.

We also observed that more taste buds remained in the *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice by day of birth. This finding is somewhat consistent with previous reports

that *TrkB*^{-/-} mice retain a substantial number of taste buds [83], while *Bdnf*^{-/-}/*Ntf4*^{-/-} mice have almost no taste buds remaining [94]. A previous examination of *TrkB*^{-/-} mice claimed that these mice lose most of the geniculate neurons and have no remaining taste innervation to the tongue [83]. As a result the *TrkB*^{-/-} mice have provided evidence that innervation is not required for the development of taste buds. Contrary to this claim, many studies have shown that taste buds require taste fiber innervation to support their postnatal development ([114], [115], [116]). To re-examine the earlier claim that taste buds survive in the absence of innervation in *TrkB*^{-/-} mice and to address why there are more taste buds in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, we quantified innervation. These experiments led us to conclude that the remaining taste buds in *TrkB*^{-/-} mice were better innervated than for *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and there are more innervated taste buds in *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. We also observed un-innervated taste buds in both mutant genotypes, which do not normally occur. These taste bud remnants were smaller than normal taste buds, but demonstrate that in mice some keratin 8 stained profiles do not require innervation to be maintained. This finding is consistent with mouse studies where some small keratin 8 positive groups of cells remain following nerve sectioning in the mouse [117]. These un-innervated keratin 8 remnants do not have pores and may or may not differentiate into functional taste cell types. Thus, whether or not taste buds remain in the absence of innervation depends largely on how taste buds are defined. We also found that the number of these un-innervated taste buds is similar in the two mutant groups, confirming that the increase in taste bud number in the *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} mice is due to increased taste innervation in these mice. Consistent with our finding that taste buds are better innervated at birth in *TrkB*^{-/-} mice we found significantly

more taste fibers in the *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Based on these experiments, we conclude that the increased taste bud survival in *TrkB*^{-/-} mice compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} mice is due to more taste innervation remaining in the tongues of *TrkB*^{-/-} mice.

Since we found that *TrkB*^{-/-} mice have better taste innervation compared to *Bdnf*^{-/-}/*Ntf4*^{-/-} mice during development, we speculated that other receptors that respond to BDNF and/or NT4, such as truncated TrkB, may also mediate taste innervation. However, our data showed that the remaining geniculate neurons in the *TrkB*^{-/-} mice do not express any of the TrkB isoforms including the truncated TrkB isoform, indicating other mechanism(s) must be involved. We speculate that this differential effect on the taste innervation of *TrkB*^{-/-} and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice may be due to the contribution of Schwann cells. Although the TrkB receptor is not expressed in the geniculate neurons in *TrkB*^{-/-} mice, it could remain in Schwann cells of *TrkB*^{-/-} mice. Consistent with this idea we observed a large amount of TrkB-positive tissue surrounding the geniculate ganglia. If this tissue contains glial cells that express the truncated TrkB-receptor then this receptor could mediate BDNF-evoked calcium signaling ([118]) and affect axonal growth. Another possibility is that BDNF could promote Schwann cell myelination independent of TrkB ([119]). Therefore Schwann cell myelination may be more severely affected in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice than in the *TrkB*^{-/-} mice. It is known that myelination protects the integrity of axons ([120]), and impairments of myelination in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice may lead to degeneration of axons and result in less taste innervation.

Our findings showed that about one-third of the normal number of taste buds remained in the tongues in the *TrkB*^{-/-} mice, although only one-tenth of the normal number of geniculate neurons remained to innervate the tongue. The remaining number

of taste buds is surprisingly large considering the huge loss in geniculate neuron numbers. However, this is consistent with several studies that demonstrate that loss of taste buds is not directly predictable from neuron loss [89]. For example, *Ntf3*^{-/-} mice lose geniculate neurons ([103], [47] but do not lose taste buds [87]). In the *Ntf4*^{-/-} mice, roughly half of the geniculate neurons were gone, but there was only very modest loss of taste buds [89], [51]. The reason more taste buds were maintained than might be expected based on the loss of neurons is because each taste bud is innervated by multiple geniculate neurons ([121]). In the *TrkB*^{-/-} mice, about 194 (97/side) geniculate neurons remain to innervate the 31±3 remaining taste buds on the tongue. Therefore, the number of geniculate neurons in *TrkB*^{-/-} mice was still sufficient to support the additional taste buds we observed in *TrkB*^{-/-} mice compared with *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Since roughly the same number of neurons remains in the geniculate ganglion of *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice, it follows that there are sufficient neurons available in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice to maintain 31 taste buds also. So why were their fewer taste buds remaining in the tongue of *Bdnf*^{-/-}/*Ntf4*^{-/-} mice? In addition to the neuron loss, *Bdnf*^{-/-}/*Ntf4*^{-/-} mice show disrupted target innervation of remaining afferents, much like with *Bdnf*^{-/-} mice [56]. The failure of these remaining neurons to successfully reach their targets contributes to the loss of innervation to taste buds in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice.

If *TrkB*^{-/-} and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice lose approximately the same number of geniculate ganglion neurons during development, and the reduced taste innervation in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice compared to *TrkB*^{-/-} mice is due to disrupted target innervation in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, the implication of these findings is that target innervation is not disrupted in *TrkB*^{-/-} mice. Consistently, we did observe that the small number of geniculate

ganglion neurons remaining in *TrkB*^{-/-} mice, were very successful at innervating the tongue surface. These neurons completely lack all forms of the TrkB receptor, yet are better at innervating taste buds than the remaining neurons in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, indicating that they are responsive to BDNF, NT-4 or both. It is likely that other neurotrophins and their receptors are responsible for the survival of these geniculate neurons. Other neurotrophin receptors are expressed in the geniculate ganglion ([122], [123]), the most notable of which is the NT-3 receptor TrkC and the receptor for the Gdnf family of ligands, Ret [124], [125], [126]. While other neurotrophins are likely involved in the survival of these remaining neurons, BDNF and NT-4 must somehow be involved in the ability of these neurons to innervate the lingual epithelium. Among the remaining possible mechanisms is the p75 receptor, which binds BDNF and NT-4 in addition to other neurotrophins. Future studies are needed to examine how BDNF and/or NT-4 function through p75 receptor to support the survival of geniculate neurons and how TrkB interacts with p75 to regulate taste development.

CHAPTER 3

THE NEUROTROPHIN RECEPTOR P75 REGULATES GUSTATORY NEURON SURVIVAL AND MEDIATES TASTE NERVE INNERVATION THE MID-REGION OF THE TONGUE

3.1 Introduction

Neurotrophins are a group of target derived growth factors that were first identified as survival factors in sympathetic and sensory neurons. Since then they have been shown to be important regulators in many other aspects of development and function of peripheral neurons. Neurotrophins bind to two types of receptors: the tyrosine kinase receptor (Trk) and the p75 neurotrophin receptor, which is a member of tumor necrosis factor (TNF) receptor superfamily ([26]). The functions of Trk receptors after neurotrophin binding are well understood, including cell survival, proliferation, axon growth, and synapse plasticity. However, the function the p75 remains unclear. Previous studies have shown opposite roles of p75 in cell survival and apoptosis.

In the peripheral nervous system, the p75 and Trk receptors are usually co-expressed. The p75 receptor can function as a positive survival factor by enhancing Trk receptor function. For example, p75 was found to be a pro-survival in regulating TrkA function in vitro by increasing the number of high-affinity binding sites ([127], [128]). *In vivo* data showed that $p75^{-/-}$ mice lose sensory and sympathetic innervation ([129], [31], [130]). In addition, trigeminal neurons and sympathetic neurons in the $p75^{-/-}$ mice require

four folds more NGF for cell survival than wild type neurons ([129], [31]). This finding indicates mutants of the p75 receptor have lower sensitivity for neurotrophin signaling through the TrkA receptor. All of these data support the hypothesis that p75 function as a positive modulator upon Trk receptor function.

On the other hand, several studies indicate that p75 function as a pro-apoptosis factor as a result of ligand binding ([30], [131]). The examination of *p75^{-/-}* mice not only supports a pro-survival role ([129], [31], [130]) in some neuron populations, but in some other cases, supports the opposite function. For example, the number of cholinergic neurons in the basal forebrain of *p75^{-/-}* mice was significantly increased compared to wild type mice ([132], [133]). These results indicate that p75 serve as a pro-apoptosis factor in these cell populations. Observations in embryonic retina showed that p75 is required for NGF-mediated apoptosis ([30]). Specifically, NGF binding to p75 in the absence of TrkA induced apoptosis of precursor cells, while blocking both NGF and p75 with antibodies prevented apoptosis ([30]). In addition, the neurotrophin BDNF, which does not activate Trk receptors on sympathetic neurons, activates p75 to cause neuronal apoptosis [131]. The apoptosis role of p75 is also observed *in vitro*. Cultured glial cells expressing p75 are effectively killed by NGF ([134]). Similarly, cultured embryonic trigeminal neurons are killed by NGF through binding to p75 ([135]). This evidence firmly supports the hypothesis that p75 induce apoptosis by direct neurotrophin binding.

The p75 receptor also exhibits a ligand-independent pro-death role. It has been shown that temperature sensitive immortalized neural cells expressing p75 die when p75 was unbound. Also, binding by NGF or antibody inhibited cell death induced by p75 ([136]). In addition, down-regulation of p75 with antisense oligonucleotides in sensory

neurons prevents NGF-mediated survival at the stage of target innervation; however, at later embryonic developmental ages, p75 knockdown promotes the survival of neurons in the absence of NGF ([137]). This study indicates p75 may switch functions during different developmental ages. These observations taken together suggest a neurotrophin-independent apoptosis function for p75, which could be explained by a intracellular death domain in the p75 receptor analogous to the death domains in other TNF receptor family members ([138], [139]).

In addition to its function in regulating neuron numbers, the p75 receptor has also been reported to mediate axon growth ([140], [141]) and maintain innervation to peripheral targets ([31], [130]). For example, *p75^{-/-}* mice have been shown lose sensory innervation to their footpads ([31]). Removal of p75 also leads to deficits in sensory receptors. For instance, a specific receptor complex, consisting of slowly adapting type 1 (SA1) mechanosensory neurons and Merkel cells, requires p75 for its maintenance ([142], [143], [144]). In some reports, Merkel cell loss may be not due to loss of innervation, but a direct effect of p75 signaling in epithelial-derived sensory cells ([143]). Overall, p75 is important in regulating innervation as well as peripheral sensory receptors.

In the taste system, the p75 receptor is expressed in both taste bud cells ([145], [146]) and geniculate ganglion neurons ([147]). In vitro studies also showed that p75 is involved in geniculate neurite outgrowth ([55]). Mice lacking p75 showed loss of geniculate neurons and taste bud loss by adulthood ([147]). However, it remains unclear if p75 supports neuronal survival during development and at what age. It is also unknown if p75 regulation of neuron number differs depending of the presence or absence of TrkB. In addition, it would be interesting to know if p75 regulates taste nerve innervation and

taste bud formation. It is possible that p75 removal disrupts taste innervation and as a result impairs the formation of the taste bud. It is equally possible that p75 regulates taste buds in-dependent of innervation as those seen in the Merkel cells. Here we examined neuron survival, target innervation and taste bud formation in $p75^{-/-}$ and $TrkB^{-/-}/p75^{-/-}$ mice to address these questions.

3.2 Methods

3.2.1 Animals

Heterozygous $TrkB^{+/-}$ (stock no. 002544) and $p75^{+/-}$ (stock no. 002213) mice were acquired from Jackson Laboratories (Bar Harbor, Maine, USA,). The $p75^{-/-}$ embryos were obtained by breeding heterozygous mice with a target mutation of the $p75$ gene. The $p75^{-/-}$ mice were also bred with $TrkB^{+/-}$ to obtain $TrkB^{+/-}/p75^{+/-}$. $TrkB^{+/-}/p75^{-/-}$ mice were obtained by breeding $TrkB^{+/-}/p75^{+/-}$ mice with $p75^{-/-}$ mice. $TrkB^{-/-}/p75^{-/-}$ embryos were obtained by breeding $TrkB^{+/-}/p75^{-/-}$ mice. Animals were genotyped using polymerase chain reaction. Embryonic mice were obtained from time breeding of females that were examined for plugs the following morning. The day a plug was positively identified was designated as embryonic day 0.5 (E0.5). Animals were cared for and used in accordance with the guidelines of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

3.2.2 Quantification of geniculate ganglion neuron number

Embryos aged E13.5 ($p75^{-/-}$ n=3, $TrkB^{-/-}/p75^{-/-}$ n=2, and wild-type n=3), E14.5 ($p75^{-/-}$ n=3, and wild-type n=3), E16.5 ($p75^{-/-}$ n=3, and wild-type n=3) and E18.5 ($p75^{-/-}$ n=3, and wild-type n=3) were transcardially perfused with ice cold 4% phosphate-buffered paraformaldehyde (PFA). Following perfusion, embryos were post-fixed overnight in 4% PFA. Following fixation, embryo heads were dissected and moved to 70% ethanol and processed for paraffin embedding. Geniculate ganglion neurons were visualized by class III β -tubulin (TUJ-1) antibody as previously described ([52]). Briefly, serial sections (5 μ m) of paraffin embedded embryos were collected on SuperFrost Plus slides (Fisher Scientific). Paraffin was removed by immersion in Citrisolv overnight. Following rehydration and endogenous peroxidase blocking, slides were treated for antigen retrieval in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, dH₂O; pH 6). Sections were washed in PBS and blocked for 1 hr in blocking solution (PBS, 5% goat serum, 0.25% Triton X-100), and were incubated overnight in mouse anti- β -III tubulin antibody (1:500, Covance, Princeton, NJ, USA; catalog #MMS-435P) in blocking solution. On the following day, sections were washed and incubated for 1.5 h in biotinylated anti-mouse secondary antibody (1:250, Vector Laboratories, Burlingame, CA, USA; #BA-2000) in blocking solution, and visualized with an ABC diaminobenzidine reaction kit (Vector Laboratories, Burlingame, CA, USA; #PK-6200).

For the measurement of geniculate neuron number, the TUJ-1 antibody was used to identify and count neuronal profiles in sections where the nucleus was visible. Neuronal profiles were counted in six representative sections per ganglion. The volumes of geniculate ganglion in these six sections were measured. The volume of the total

geniculate ganglion volume was also measured. To estimate the total volume of the geniculate ganglion, the size of geniculate ganglion in each section was measured and multiplied by the section thickness (5 μ m) to derive the volume of the single section; these volumes were added to derive the total volume for the entire ganglion. The total number of neuronal profiles of the entire ganglion was estimated as the product of the number of profiles per volume of the counted section \times the total volume of the entire ganglion. The total number of neurons per ganglion was estimated by multiplying the number of total neuron profiles by a correction factor to compensate for the presence of a nucleus in multiple sections (Abercrombie, 1946). The correction factor was calculated according to the formula: $N = n \times [T/(T \times D)]$, where N is the estimated total number of neurons, n is the number of nuclear profiles, T is the measured section thickness, and D is the average diameter of the nuclei ([52]).

3.2.3 Quantification of taste bud number and taste bud innervations

Mice at day of birth (*p75*^{-/-} n=5, *TrkB*^{-/-}/*p75*^{-/-} n=3, and wild-type n=4) and mice at p10 were (*p75*^{-/-} n=3 and wild-type n=3) were anesthetized and transcardially perfused with ice cold 4% PFA. The front of the tongue containing the fungiform field was separated and post-fixed in 4% PFA for two hours. Tongues were then placed in 30% sucrose overnight as cryoprotectant. The following day, tongues were embedded in OCT (Sakura Finetek USA, Inc., #4583). Serial sagittal sections of the tongue (25 μ m) were collected onto SuperFrost Plus slides (Fisher). For antigen retrieval, sections were heat

dried overnight, rehydrated, placed into citrate buffer (pH 6.0), heated for 15 min in a boiling water bath, and incubated for 10 min at RT.

For primary antibodies, rat anti-Troma1 antibody (1:50 Developmental Studies Hybridoma Bank) and rabbit anti-P2X3 antibody (1:500, Millipore, #AB5895) were used to label taste buds and taste fibers. Secondary anti-rat Alexa 488 (green) and anti-rabbit Alexa 555 (red) antibodies (1:500, Molecular Probes) were used to visualize taste buds and taste fibers respectively. The sections were examined in order, and the taste buds were followed across sections so that each taste bud was only counted once.

To measure the innervation within the taste bud, confocal stacks of optical sections with a Z step of 0.5 were imaged of 3-5 taste buds from every mouse for each genotype (*p75^{-/-}* n=5, and wild-type n=4) and then analyzed by ImageJ (<http://rsbweb.nih.gov/ij/>). The area occupied by the taste bud in each image section was measured; areas were summed and multiplied by section thickness (0.5um) to calculate taste bud volume. The area occupied by P2X3 positive staining within the outlined taste bud was also measured in each optical section; these areas were summed and multiplied by section thickness (0.5µm) to measure the volume of innervation within the taste bud. The percentage of the taste bud that was occupied by innervation was determined by dividing the volume of P2X3 label by the volume of the Troma1 label.

3.2.4 Geniculate ganglia labeling using DiI

Embryos at ages E14.5, E16.5 and E18.5 were anesthetized and transcardially perfused in ice cold 4% (PFA). Tongues were post fixed in 4% PFA overnight. DiI labeling was performed as described previously ([50]). Embryos were incubated at 37°C for 2–8 weeks depending on the age of the embryo. The tongue was then dissected, examined, and photographed using a fluorescent dissecting microscope (Leica MZFL) equipped with a camera (QImaging CE). Images were collected from tongues of *p75^{-/-}* and wild-type mice at the following ages: E14.5 (*p75^{-/-}* n=3 and wild-type n=3), E16.5 (*p75^{-/-}* n=6 and wild-type n=5) and E18.5 (*p75^{-/-}* n=4 and wild-type n=6).

3.2.5 Data analysis

The total neuron numbers were compared between genotypes on embryonic days E13.5, E14.5 E16.5, and E18.5 using a two-way analysis of variance (ANOVA). The taste bud number and volume, number of innervated data were compared using one-way analysis of variance (ANOVA). The alpha levels were set at $p < 0.05$ for all statistical comparisons. The data were described as mean \pm S.E.M. in the text and figures.

3.3 Results

3.3.1 The p75 receptor does not mediate neurotrophin survival/death during early development

Mice lacking the p75 receptor lose about 25% of geniculate neurons by adulthood ([147]). However it remains unclear if $p75^{-/-}$ mice lose neurons during embryonic ages and if so when this loss happens. Geniculate neuron survival depends on both BDNF and NT-4. It has been shown that $Ntf4^{-/-}$ mice start to lose geniculate neurons at E11.5 while $Bdnf^{-/-}$ mice start neuron loss at E13.5 ([51,52]). In chapter 2, I showed that most of geniculate neurons are lost in $TrkB^{-/-}$ and $Bdnf^{-/-}/Ntf4^{-/-}$ mice by E13.5, suggesting that TrkB is the primary receptor for BDNF and NT-4 in regulating neuron survival. However, it is possible that either BDNF or NT-4 can also function through p75 receptor to support neuron survival. To test this hypothesis, we examined the geniculate neuron number in $p75^{-/-}$ and $TrkB^{-/-}/p75^{-/-}$ mice at E13.5. We reasoned that if the p75 receptor mediates geniculate neuron survival by the binding of BDNF or NT-4, we would see neuron loss in $p75^{-/-}$ mice by E13.5, and we also would see a decrease in neuron number of $TrkB^{-/-}/p75^{-/-}$ mice compared to the $TrkB^{-/-}$ mice at this age.

Using anti- β -III tubulin antibody (TUJ-1), geniculate neurons were easily identified due to their clear nucleus and dark cytoplasm (Figure 13). We counted the number of geniculate neurons in $p75^{-/-}$ mice, $TrkB^{-/-}/p75^{-/-}$ mice and wild type mice (Figure 14) and compared them with data from $TrkB^{-/-}$ mice (Chapter 2). We found that, compared to the wild type mice, there was no significant neuron loss in $p75^{-/-}$ mice. In addition, $TrkB^{-/-}$ mice and $TrkB^{-/-}/p75^{-/-}$ mice lose most of their geniculate neurons, and

there is no significant difference between the number of remaining neurons in *TrkB*^{-/-} mice compared with *TrkB*^{-/-}/*p75*^{-/-} mice. These results indicate that p75 does not function by mediating BDNF and NT-4 to support taste neuron survival during early development. In addition, removing p75 along with TrkB neither enhances the loss of neurons seen in TrkB knockouts, nor rescues the loss of neurons in TrkB knockouts. Taken together these data indicate that p75 is neither a pro-survival nor a pro-death factor for taste neurons during early development.

3.3.2 Geniculate neurons were lost in *p75*^{-/-} mice from E14.5 to E18.5

Since *p75* mutant mice lose about 25% geniculate neurons during adulthood and *p75*^{-/-} mice have similar neuron number compared to wild type at E13.5, we examined a later age, E18.5, to ask if the neuron loss occurs before birth. We found that compared to wild type mice, *p75*^{-/-} mice lose about 36% of their geniculate neurons at E18.5 (Figure 16). This result indicated that the effect of p75 in regulating neuron number is an embryonic effect that is maintained until adulthood. To address when this effect occurs, we examined neuron loss in *p75*^{-/-} mice at earlier ages E14.5 and E16.5. At E14.5, geniculate ganglion was smaller in *p75*^{-/-} mice (Figure 15), and geniculate neuron number in the wild type mice and *p75*^{-/-} mice are 528±69 and 412±21, respectively. At E16.5, geniculate neuron number in the wild type mice and *p75*^{-/-} are 435±56 and 254±37, respectively. There are significantly fewer neurons remaining in the *p75*^{-/-} mice compared to the wild type mice at both ages ($p < 0.01$). Taken together with previous neuron counting, we conclude that *p75*^{-/-} mice start to lose geniculate neurons at E14.5 and this

loss continues until later ages. Since E14.5 is the age taste nerves start to innervate peripheral targets ([56]) and neuron number is controlled by the amount of trophic support from the peripheral targets, we reasoned that the loss of geniculate neurons seen in $p75^{-/-}$ mice may be due to disrupted innervation to the peripheral targets.

3.3.3 Taste nerves lose taste innervation to the mid-region of tongue in $p75^{-/-}$ mice

To address the possibility that neuron loss in $p75^{-/-}$ mice may be due to disrupted innervation, we used DiI-labeling of the geniculate ganglion to examine taste innervation in the $p75^{-/-}$ mice during development. In the wild type mice, at E14.5, chorda tympani fiber bundles are visible from the dorsal surface of the tongue and formed a bulblike termination called a “neural bud” ([42]). Chorda tympani fibers reach the entire medial-to-lateral epithelium surface of the tongue and neural buds are visible across the dorsal surface of the tongue by E14.5. In contrast, in the $p75^{-/-}$ mice, there were only a few neural buds present at the dorsal surface of the tongue (Figure 17 B). The neural buds in the $p75^{-/-}$ mice at E14.5 were located at both anterior and posterior region of the tongue. A few neural buds were visible at the tip of the dorsal tongue. Since innervation of the tongue proceeds from caudal to rostral during development ([148]), neural buds at the tongue tip suggest that axonal growth and targeting is not delayed in the $p75^{-/-}$ mice.

To determine if the loss of taste innervation in the $p75^{-/-}$ mice continued through the later developmental ages, we examined the innervation pattern in $p75^{-/-}$ mice at E16.5 and E18.5. The loss of innervation in the $p75^{-/-}$ mice persisted during the later ages. In the $p75^{-/-}$ mice, at E16.5, the chorda tympani innervation pattern was similar to E14.5.

Compared to the wild type mice, the innervation to the tip of the dorsal tongue looks similar. However, at the posterior part of the dorsal tongue surface, there were fewer neural buds around the middle region of the tongue in the $p75^{-/-}$ mice. In fact, fiber branches do not even enter the tongue mid-region in $p75^{-/-}$ mice. This loss of neural buds to a specific region of the tongue continued to a later age, E18.5. At E18.5, the neural bud number in the $p75^{-/-}$ mice was severely reduced compared to wild type littermates (Figure 17 F). The density of neural buds was highest at the tip of the tongue. At the posterior part of the tongue, neural buds were only seen at the very lateral edges of the tongue surface. We sectioned the tongue and examined the innervation to the dorsal surface, these results confirmed that the tip of the targets of the tongue in the $p75^{-/-}$ mice were innervated, however, $p75^{-/-}$ mice lose innervation to the posterior part of the tongue (Figure 18). Taken together, these results show that $p75^{-/-}$ mice lose innervation to the medial-caudal area of the tongue during development.

3.3.4 Taste buds are lost and taste bud innervation is reduced at P0 in $p75^{-/-}$ mice

It has been reported that p75 is important for maintaining the normal number of circumvallate taste buds in adulthood ([147]). The same report found that $p75^{-/-}$ mice lose about 36% of their fungiform papillae and 25% of their fungiform taste buds by adulthood. However, it remains un-determined if p75 regulates fungiform taste buds during development. Here we used Troma-1 and P2X3 anti-bodies to label taste buds and taste nerves of $p75^{-/-}$ mice at P0 to determine how p75 regulates fungiform taste bud formation. In wild type mice all taste buds were innervated; however, we found both

innervated and un-innervated taste buds in the tongue sections at P0 in the $p75^{-/-}$ mice (Figure 19 C). This finding is consistent with embryonic innervation patterns, which show that innervated taste buds are near the tip of the tongue while the un-innervated taste buds are located at the posterior area of the tongue. We measured the volume of the two types of taste buds and found that innervated taste buds were significantly larger than un-innervated taste buds. We also counted the number of taste buds in the $p75^{-/-}$ mice (Figure 20). Compared to the wild type mice, the number of taste buds in the $p75^{-/-}$ mice was significantly decreased (wild type=103±7, n=5, $p75^{-/-}$ =60±4, n=4), suggesting that proper innervation is required for the normal development of taste buds. Mutation of the $p75$ receptor disrupted innervation to the taste bud causing a loss of both taste bud number and volume (Figure 21).

It is well established that innervation is required to support taste buds during development ([77], [78]); therefore, it is likely that uninnervated taste buds would be lost at later ages. To determine if those un-innervated taste buds remain until later developmental ages, we counted the taste bud number in the $p75^{-/-}$ mice at P10. Compared to the wild type mice (116±7), there are about 75±2 taste buds in the $p75^{-/-}$ mice. Surprisingly, no additional taste buds were lost in $p75^{-/-}$ mice at P10 (35%) compare to P0 (42%), suggesting there may be other mechanism/s that support the survival of un-innervated taste buds in $p75^{-/-}$ mice (Figure 22).

BDNF and NT-4 have been shown to promote apoptosis of sympathetic neurons through $p75$ in the absence of $TrkB$ ([149], [131]) However, since neuron number is not rescued in $TrkB^{-/-}/p75^{-/-}$ mice compared to the $TrkB^{-/-}$ mice, this does not appear to be the case for the developing taste system. Surprisingly, we did observe a rescue of taste bud

number in the *TrkB*^{-/-}/*p75*^{-/-} mice compared to *TrkB*^{-/-}. At P0, *TrkB*^{-/-}/*p75*^{-/-} mice retained more taste buds than the *TrkB*^{-/-} mice, more interestingly, Troma1/P2X3 staining experiments showed that these remaining taste buds are P2X3 negative (Figure 19H), there was no taste innervation to these taste buds in the *TrkB*^{-/-}/*p75*^{-/-} mice. These results indicated that p75 may function to promote apoptosis in the taste bud in the absence of innervation; this finding is consistent with the finding that un-innervated taste buds remain in the tongues of P10 *p75*^{-/-} mice.

3.4 Discussion

Geniculate neurons depend equally on BDNF and NT-4 for survival, however, it happens at different times and via different mechanisms ([46], [52], [51]). NT-4 derived from the ganglion and along the projection pathway supports geniculate neuron survival as early as E11.5, via a caspase-3 independent mechanism. From E13.5 to E18.5, BDNF acts as a classic target derived survival factor to prevent cell death via a caspase-3 dependent pathway. Most of the geniculate neurons are lost in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice by E13.5. There is no significant difference in the remaining neuron number between *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice, suggesting that TrkB acts as the primary receptor for taste neuron survival during the early developmental ages. However, a sub population of taste neurons remained in the *TrkB*^{-/-} mice, and these neurons succeed in innervating and supporting the taste bud formation. To test if the p75 receptor supports the survival of this sub-population of neurons, we examined the geniculate neuron number in *p75*^{-/-} mice and *TrkB*^{-/-}/*p75*^{-/-} mice at E13.5. We found that *p75*^{-/-} mice do not

lose taste neurons compared to the wild type, indicating that p75 does not promote survival. In addition, we found that there was no increase of neuron loss in *TrkB*^{-/-}/*p75*^{-/-} mice compared to *TrkB*^{-/-} mice. This finding indicates that p75 is not required for the survival of the TrkB-independent subpopulation of taste neurons.

The p75 receptor has been shown to promote neuronal apoptosis when the Trk receptors are absent ([30], [131], [150]). For example, early embryonic endogenous NGF causes death of retinal neurons that express p75 but not TrkA ([30]). BDNF activates p75 to induce apoptosis of sympathetic neurons ([131]). Loss of both TrkA and p75 rescues part of the neuronal death in DRG neurons caused by TrkA removal, which suggests that apoptosis is in part due to the pro-death role of p75 ([151]). However, we didn't observe the same effect for the geniculate neurons. At E13.5, *TrkB*^{-/-}/*p75*^{-/-} mice have similar geniculate neuron numbers as *TrkB*^{-/-} mice. This result indicates a possible different role of p75 in taste neurons than in DRG neuron survival ([151]). This difference may be due to differences in Trk expression. Specifically, the mutants of TrkA and TrkC caused apoptosis for the sympathetic and sensory neurons, but TrkB may not since it is less involved in those populations. In taste neurons, TrkB is the major receptor that regulates neuron survival, so that may be why *TrkB*^{-/-}/*p75*^{-/-} mice did not show a rescue in neuron loss as those seen in *TrkA*^{-/-}/*p75*^{-/-} mice. Also, the study only measured the number of caspase-3 positive DRG neurons, instead of comparing the actual DRG neuron number in *TrkA*^{-/-} and *TrkA*^{-/-}/*p75*^{-/-} mice. It is possible the remaining DRG neurons in the *TrkA*^{-/-}/*p75*^{-/-} mice is similar to *TrkA*^{-/-} mice, which is in line of our findings. Overall, our results indicate that p75 does not cause neuronal apoptosis when TrkB is absent during taste neuron development.

Although the p75 receptor does not support taste neuron survival during early development, we do find that *p75^{-/-}* mice lose geniculate neurons by E14.5 and the loss continues until E18.5. This is after normal neurotrophin dependence begins so it is not likely that p75 functions by mediating the effects of neurotrophins on neuron survival. Instead, since taste nerves reach the tongue epithelium and innervate fungiform placodes at E14.5 ([42], [56]), the reduced target innervation in *p75^{-/-}* mice results in reduced neurotrophin support from the peripheral targets. This could lead to the loss of taste neurons starting from E14.5. Consistent with our finding, it has been shown that p75 is required for normal axon growth to distant targets in other systems ([31], [130]). We found that at as early as E14.5, there were fewer neural buds innervating the fungiform placodes in *p75^{-/-}* mice. The taste nerves reach the tip of the tongue which is normally the last part of the tongue innervated ([148]), this means that taste nerve growth is reduced rather than delayed in the *p75^{-/-}* mice. In addition, there was only a 30% loss in geniculate neuron number; however, we observed much more severe loss of taste innervation across ages from E14.5 to E18.5. Given that the impact on innervation pattern appears more substantial than the neuron loss, it is more likely that disrupted innervation caused reduced number of neurons than the other way. Regardless our findings demonstrate that p75 functions in supporting taste nerve growth to fungiform placodes, particularly in the tongue mid-region.

The number of neural buds in *p75^{-/-}* mice was decreased, and most of the taste nerve endings were missing near the middle and caudal area of the tongue. This pattern is very unique. One possible explanation of this specific innervation pattern is that p75 modulates the activity of Semaphorin3A (Sema3A). Sema3A is expressed in tongue

epithelium as early as E13.5 and continues to at least E18.5, and is evident in those fungiform papillae that are penetrated by taste nerves ([64]). *Sema3A* repels geniculate ganglion axons *in vitro*, and *Sema3A* mutant mice showed increased taste innervation to the middle line of the tongue [54]. In addition, these repellent effects of *Sema3A* were neurotrophin dependent, both BDNF and NT-4 stimulated taste nerve outgrowth were repelled by *Sema3A* during development ([63]). Furthermore, DRG neurons in *p75^{-/-}* mice are hypersensitive to the *Sema3A*, indicating that *p75* is an important modulator of *Sema3A* activity ([152]). Lastly, *Sema3F*, which is also expressing in the tongue, may also be involved in loss of taste innervation in the *p75^{-/-}* mice due to its repellent effect of NT-4 dependent neurite outgrowth ([63], [55]).

We observed an increased taste bud number in *TrkB^{-/-}/p75^{-/-}* mice compared to *p75^{-/-}* mice. These remaining taste buds in *TrkB^{-/-}/p75^{-/-}* mice are not innervated; indicating the increase of taste bud number is not due to better innervation from the taste neurons. Studies in Merkel cells have shown that *p75* may function in the survival of peripheral targets independent of nerve innervation ([143]). In addition, *TrkB* expression is almost undetectable in tongue epithelium ([153]), so in this location *p75* is functioning independently of *TrkB*. One possible explanation of this rescue in taste bud number is *p75* positively regulates *p53*, *Bax*, caspase-2 signaling death pathway. It has been shown that *p53*, *Bax*, caspase-2 are involved in mouse circumvallate taste cell death ([154]). In addition, it has been shown that *p75* regulates *p53* associated sympathetic neuron death ([155]). Thus, in the absence of *p75*, the *p53*-*Bax*-caspase-2 death pathway could be disrupted, which results in the rescued survival of the taste bud cells.

CHAPTER 4

GENERAL DISCUSSION

The Trk receptors (TrkA, TrkB, TrkC) and the p75 receptor regulate the physiological outcomes of neurotrophins. Typically, the Trk receptors mediate the classical survival and growth properties of the neurotrophins, while the p75 receptor has been shown to regulate multiple physiological functions. In taste system, BDNF and NT-4 are two major neurotrophins regulating taste neuron survival, taste innervation, and taste bud development. BDNF and NT-4 are expressed at different times and locations in the taste system, and are responsible for differential aspects of taste development. However, the receptor mechanisms of neurotrophins regulating taste development were previously barely examined. In addition, the biological effects of p75 vary dramatically in different systems and have not yet been examined systematically in taste development. My studies of TrkB and p75 mutants provided direct evidence for how these receptors contribute to taste system development, and will lead to potential mechanisms for the functions of TrkB and p75.

The numbers of taste neurons are equally controlled by BDNF and NT-4 during development. By E13.5, *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice lose most of their geniculate neurons. There is no significant difference in the neuron number between *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice, indicating that TrkB is the primary receptor for taste neuron survival. In addition, at E13.5, there is no neuronal loss in *p75*^{-/-} mice and there is no

additional neuronal loss in *TrkB*^{-/-}/*p75*^{-/-} mice compared to *TrkB*^{-/-} mice. These results together confirmed that TrkB functions as the receptor that mediates geniculate neuron survival, and p75 does not function as a regulator to promote BDNF-TrkB and/or NT-4-TrkB survival signaling. On the other hand, we did not observe a rescue in the geniculate neuron number in *TrkB*^{-/-}/*p75*^{-/-} mice compared to *TrkB*^{-/-} mice, suggesting that p75 does not regulate taste neuron apoptosis when TrkB is absent. This finding is opposite to the findings in the DRG neurons where *TrkA*^{-/-}/*p75*^{-/-} mice have reduced caspase-3 positive neurons compared to *TrkA*^{-/-} mice ([151]), demonstrating that p75 removal decreases TrkA mediated cell death. This difference may be due to differences in the function of different Trk receptors. In this study, it was found that p75 kills neurons in the absence of TrkA and TrkC, but not in the absence of TrkB, suggesting a very different role of TrkB than the other two receptors.

Although our results validated that TrkB as the primary receptor for taste neuron survival, we do observe a small subpopulation of geniculate neurons remaining in *TrkB*^{-/-} mice. In addition, these neurons succeed in innervating the peripheral targets. Our results suggest these neurons are not truncated-TrkB dependent, since this subpopulation was negative for both full-length and truncated-isoforms of TrkB. They are also not p75-dependent, because the full length p75 was shown not to regulate their survival. However, other isoforms of p75 receptor may be involved. A mRNA splice variant of p75 that lacks exon3, which encodes the ligand binding domain, has been reported ([156]). It is possible that this alternatively spliced form of p75 may contribute to the survival of these remaining taste neurons in the *TrkB*^{-/-} mice. Alternatively, other Trk receptors, such as TrkA, might be involved since they were found to be expressed in the

geniculate neurons in a limited way ([123]). It was also shown that TrkA responds to NT-4 in fibroblasts ([157]), so it is possible that TrkA mediates part of the NT-4 survival response in taste neurons. If so, some of those NT-4 dependent neurons could survive through TrkA in the *TrkB*^{-/-} mice. However, if this is true, it is difficult to explain why there was the same number of taste neurons left in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice at E13.5. Since if TrkA mediates those NT-4 dependent neuron survival, those neurons would be lost in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice but not in *TrkB*^{-/-} mice, and we would see less neurons in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice than *TrkB*^{-/-} mice. One possible explanation is that NT-3 functions through TrkB to support another subpopulation in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Taken together, although *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice have similar taste neuron number at E13.5, those remaining neurons have different neurotrophin and/or neurotrophin receptor dependence. However, these possible dependences are subtle. Since most of the neurons in *TrkB*^{-/-} mice and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice are lost, we conclude that the majority of geniculate neurons depend on BDNF and/or NT-4 for survival, and these two neurotrophins mediate survival through TrkB-signaling, but not p75-signaling.

We observed different amounts of taste innervation and numbers of taste buds in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice. Since neuron number is the same, but target innervation and taste bud number are different in *TrkB*^{-/-} mice and *Bdnf*^{-/-}/*Ntf4*^{-/-} mice, we reasoned that BDNF and/or NT-4 function through another receptor for mediating successful target innervation. Chapter 3 study supports this hypothesis. At E14.5, target innervation was severely disrupted in *p75*^{-/-} mice, indicating a target innervation role for p75. In addition, in *p75*^{-/-} mice, taste neuron number only decreased 22% compared to wild type mice. Considering *Ntf4*^{-/-} mice lose half of their geniculate neurons but the

target innervation is barely effected, we reasoned that the number of remaining neurons in $p75^{-/-}$ mice was sufficient for normal target innervation. So the loss of taste innervation in $p75^{-/-}$ mice could be a direct effect of p75 in taste nerve growth and targeting, and not due to neuron loss. To confirm this hypothesis, taste innervation in $p75^{-/-}$ mice at E13.5 needs to be examined. If taste innervation is disrupted in $p75^{-/-}$ mice at E13.5, since there was no loss of taste neuron number at this age, targeting disruption must due to the loss of p75 regulation of nerve growth and targeting. In addition, my reasoning is also in line with the findings in Chapter 2. If my hypothesis is true, the remaining neurons in $TrkB^{-/-}$ mice would rely on BDNF or NT-4 functioning through p75 for their targeting. Therefore, in $Bdnf^{-/-}/Ntf4^{-/-}$ mice, since both neurotrophins are mutated, the targeting role of p75 would be disrupted in those few neurons that remain. My observation of increased target innervation in $TrkB^{-/-}$ mice compared with $Bdnf^{-/-}/Ntf4^{-/-}$ mice is consistent with this idea. Furthermore, although geniculate neuron number is the same in the $TrkB^{-/-}/p75^{-/-}$ mice as those in $TrkB^{-/-}$ mice, if my hypothesis is true, the hybrid mutants of both TrkB and p75 should lose more innervation to the taste bud compared to $TrkB^{-/-}$ mice. This was confirmed by our observation. At P0, few taste buds in $TrkB^{-/-}/p75^{-/-}$ mice are innervated, indicating that p75 is important for taste innervation. Taken together, our results indicate that p75 is required for the development of taste innervation patterns.

My findings suggest that the number and size of taste buds are controlled by both neural innervation and epithelial signaling. In chapter 2, I found that more taste buds are developed in $TrkB^{-/-}$ mice than $Bdnf^{-/-}/Ntf4^{-/-}$ mice. This increase in number of taste buds in $TrkB^{-/-}$ mice is due to increased taste innervation in those mice compared to $Bdnf^{-/-}/Ntf4^{-/-}$ mice. This finding supports the classic view: the induction of taste buds relies on

neural innervation. However, in chapter 3, I found that *TrkB*^{-/-}/*p75*^{-/-} mice have more taste buds compared to *TrkB*^{-/-} mice. Interestingly, those taste buds in *TrkB*^{-/-}/*p75*^{-/-} mice are not innervated. This finding may support the idea that taste bud induction is nerve-independent. However, it is also possible that those uninnervated taste buds in *TrkB*^{-/-}/*p75*^{-/-} mice are innervated initially during early embryonic ages. But by the age we examined the innervation (P0), they have already been lost. Examination of target innervation in *TrkB*^{-/-}/*p75*^{-/-} mice at an earlier age is needed to eliminate this possibility. In addition, if p75 have a possible function to kill taste buds as I reasoned from the results of *TrkB*^{-/-}/*p75*^{-/-} mice, there would be an increase in taste bud number in *p75*^{-/-} mice. However, *p75*^{-/-} mice have fewer taste buds than wild type mice because of decreased innervation. This indicates that in the *p75*^{-/-} mice, the loss of neural innervation is likely to predispose these taste buds to die even if the mutant of p75 may lead to an increase in taste bud number. Furthermore, in *p75*^{-/-} mice, innervated taste buds are much larger than un-innervated taste buds indicating that, although taste buds can form in the absence of innervation, development of the full complement of taste cells and normal taste bud morphology requires neural innervation.

My findings indicate TrkB and p75 have distinct roles in regulating taste development, which could be mediated by different signaling pathways. A recent bioinformatics study ([158]) provided an overview of a network map of BDNF-TrkB and BDNF-p75 signaling, which summarized more than one hundred downstream molecules that are involved in TrkB and/or p75 function. The major pathways activated by the TrkB receptor are Ras/mitogen-activated protein (MAP) kinase cascades, Rac/cdc42 pathways, PI3 kinase/Akt pathways, and PLC- γ 1-dependent generation of IP3 and

diacylglycerol pathways ([159]). Activation of Ras and downstream signals are required for normal neuronal differentiation and survival ([160], [161], [162], [163], [164], [165]). In addition, activation of PI3-kinase initiates the major pathways through which survival signals are conveyed ([166], [167]). These signaling pathways could contribute to the neuronal survival role of TrkB receptor activated by BDNF/NT4 in the geniculate ganglion.

My results also indicate that p75 primarily mediates taste nerve growth and targeting. In addition, p75 may directly regulate taste bud development in the absence of neural innervation. The diverse signaling pathways of p75 may contribute to these effects. Activation of p75 receptor leads to nuclear factor- κ B (NF- κ B), Jun kinase and other signaling pathways to promote apoptosis ([168], [169]), which are possibly involved in the taste bud development. In addition, neurotrophins binding to p75 eliminate p75-dependent activation of RhoA which stimulates neurite outgrowth ([140]), suggesting a possible mechanism of p75 regulate taste innervation.

Furthermore, the physiological properties of TrkB and/or p75 mutants could be the overall outcome of these mixed mechanisms. Interactions between p75 and TrkB receptor signaling could be facilitated by assembly of multi protein complexes that interact with both receptors ([170]). Future efforts focusing on sorting out these signaling molecules will no doubt shed interesting new light on the functions of these receptors.

FIGURES

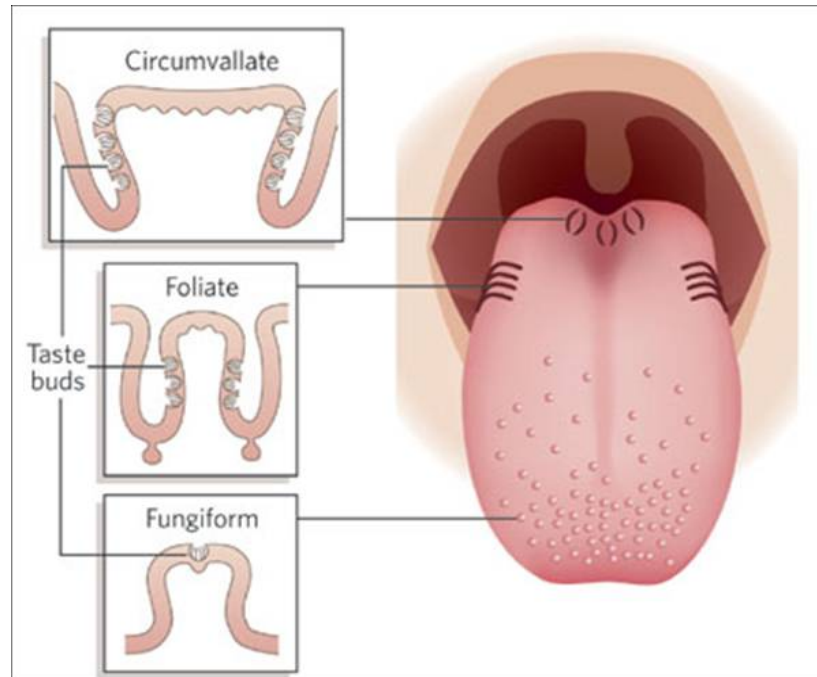


Figure 1. Three types of papillae on the tongue.

Taste receptor cells, distributed across different papillae. Fungiform papillae contain one or a few taste buds and are found in the anterior two-thirds of the tongue. Foliate papillae are present at the posterior lateral edge of the tongue and contain a dozen to hundreds of taste buds. Circumvallate papillae are found at the very back of the tongue and contain hundreds of taste buds. (Chandrashekar et al., 2006)

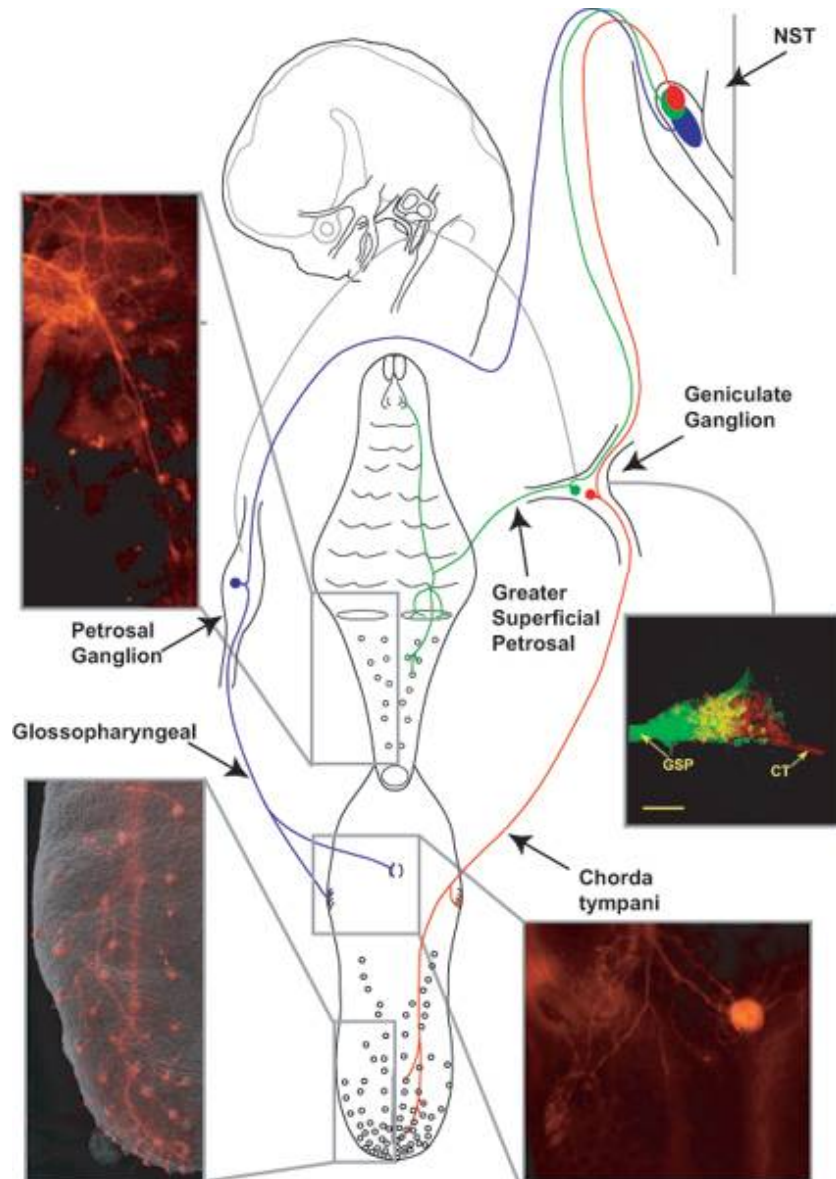


Figure 2. An overview of the basic neuroanatomy of the gustatory system.

A cartoon of geniculate neurons innervating the tongue (red) and the palate (green) and petrosal neurons innervating the tongue (blue) are shown innervating peripheral taste bud containing regions and the rostral nucleus of the solitary tract (NST). (Krimm, 2007)

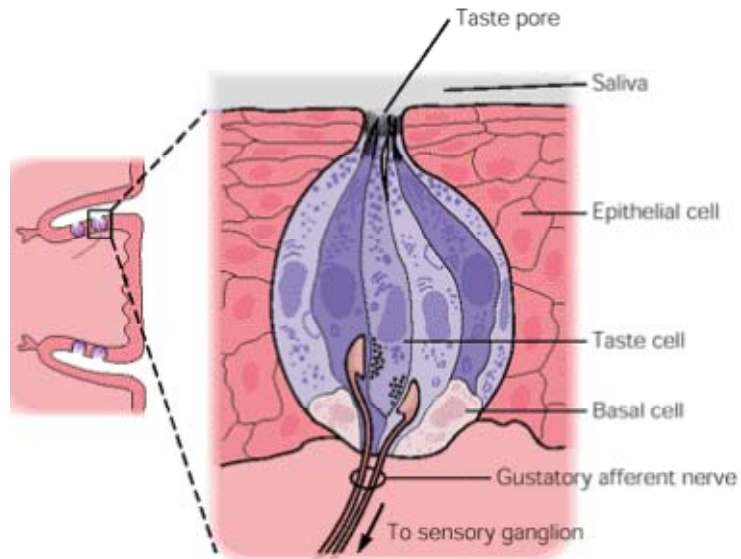


Figure 3. Four types of taste cells in mammals.

Three types of taste cells in each taste bud (light cells, dark cells, and intermediate cells) may represent different stages of differentiation or different cell lineages. Taste stimuli, detected at the apical end of the taste cell, induce action potentials that cause the release of neurotransmitter at synapses formed at the base of the taste cell with gustatory fibers that transmit signals to the brain. Basal cells, small round cells at the base of the taste buds, are thought to be the stem cells from which other cells are derived. (Principle of Neuroscience)

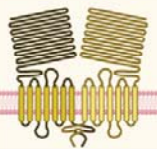

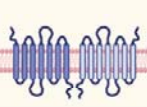
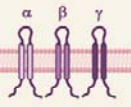
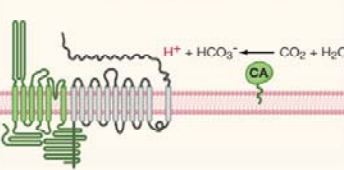
Mammalian taste receptors and cells				
Umami	Sweet	Bitter	Sodium	Sour and carbonation cells
				
T1R1+T1R3	T1R2+T1R3	~30 T2Rs	ENaC	PKD2L1 CA IV
L-glutamate L-amino acids glycine L-AP4	Sugars Sucrose, fructose, glucose	Cycloheximide (mT2R5)	Low NaCl Sodium salts	Acids Citric acid Tartaric acid HCl
Nucleotide enhancers IMP, GMP, AMP	Artificial sweeteners saccharin, acesulfame K aspartame, cyclamate	Denatonium (mT2R6, ht2R4)		Carbonated drinks
	D-amino acids D-alanine, D-serine, D-phenylalanine	Salicin (ht2R16)		
	Glycine	PTC (ht2R38)		
	Sweet proteins Monellin, thaumatin	Saccharin (ht2R43, ht2R44)		
		Quinine strychnine atropine		

Figure 4. Mammalian Taste Receptors, Cells, and Ligands.

Detection of the gustatory world is mediated by several distinct classes of taste receptors and taste receptor cells. Sweet and umami compounds are sensed by T1R heterodimers, while bitter compounds activate T2R receptors. Salt is detected via several mechanisms, one of which is thought to rely on the sodium channel ENaC. Sour-sensing cells are defined by the expression of PKD2L1, whereas gustatory responses to carbonation are mediated by the membrane-tethered carbonic anhydrase CA IV. (Yarmolinsky et al., 2009)

Table 1. Neuronal losses in the sensory system of neurotrophin- and neurotrophin receptor-deficient mice.

	trkA	NGF	trkB	BDNF	NT-4/5	BDNF/NT-4	trkC	NT-3	trkB/trkC	BDNF/NT3
<u>Spinal</u>										
Neuron loss	70–80%	70–80%	30%	35%	5%		20%	60%	40%	
Modality lost	nociceptive, thermoceptive, and AM mechanoreceptors		Meissner	Meissner	D-hair afferents		proprioceptive	proprioceptive, D-hair afferents and SA mechanoreceptors		
<u>Timing</u>										
E13	70–80%	70–80%	0%	0–30%	0%	0%	20%	60%	–	
P0	70–80%	70–80%	0%	0–30%	0%	0%	20%	60%	40%	
P15	nonviable	nonviable	30%	35%	–	–	20%	nonviable	nonviable	
adult	nonviable	nonviable	nonviable	nonviable	5%	–	20%	nonviable	nonviable	
<u>Cranial</u>										
TRG	70%	–	30%	30–45%	0%		20%	60%		
TMN	–	–	–	30–40%	10%		50%	60%		
Geniculate	–	–	95%	50%	50%		15–20%	30%	98%	65%
Vestibular	–	–	60–85%	85%	10%	100%	15–30%	20–30%	100%	100%
Cochlear	–	–	20%	10%	–	–	55–70%	85%	100%	100%
Nodose	–	–	95%	45–60%	55–60%	85–95%	15–20%	30–40%	95%	60–70%

Table 1. Neuronal losses in the sensory system of neurotrophin- and neurotrophin receptor-deficient mice.

Neuronal losses in the sensory system of mice carrying targeted mutations in genes coding for the different neurotrophins and their receptors. Available information on neuronal losses in double homozygous mutant mice is also included. Neuronal losses are expressed as the percentage of neurons lost in the mutant compared with wild-type controls. In the case of DRG, the modalities lost and the approximate timing for the deficit are indicated. TRG, trigeminal ganglion; TMN, trigeminal mesencephalic nucleus. See text for original references. (Kirstein and Fariñas, 2002)

Gustatory ganglion	Placode formation	Placode delamination migration	Initial axon outgrowth	Peak cell production	Axons reach tongue	Peak cell death	Target innervation
Mouse	E8.5	E9.5	E9.5	E10.5	E12	E14.5	E14-15
Rat	E9.5-10	E11	E11.5	E12.5	E13.5	E16.5	E16.5
Tongue and taste buds	Tongue	Fungiform papillae (placode) (SEM)	Full no. of fungiform papillae	Taste bud differentiation begins	Taste pores	Full no. of vallate taste buds	
Mouse	E12	E13-13.5	E14.5	E16.5	postnatal	adult	
Rat	E13.5	E14.5-15.5	E16.5	E20.5	postnatal	adult	

Table 2. A general timetable of major morphological changes is provided for rats and mice.

The first sperm/plug positive day is considered day 0.5. Mice typically develop two days earlier than rats. The bold time points have been determined experimentally, non-bold numbers are estimated values based on this two-day difference. (Krimm, 2007)

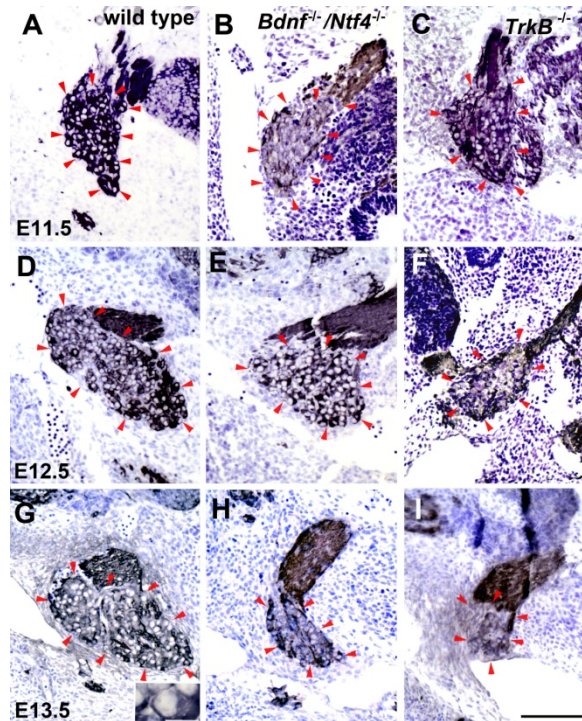


Figure 5. TUJ-1 labeled geniculate ganglion from *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice decrease in size compared to wild type mice between E11.5 and E13.5. Paraffin-embedded sections were stained with TUJ1 antibody. Geniculate ganglion volume from *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B, E, H) and *TrkB*^{-/-} mice (C, F, I) were smaller than those of wild type mice (A, D, G) starting from E12.5. At E11.5, the geniculate ganglion were similar in size in wild type mice (A), *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B) and *TrkB*^{-/-} mice (C). At E12.5, the size of geniculate ganglion appeared reduced in *Bdnf*^{-/-}/*Ntf4*^{-/-} (E) and *TrkB*^{-/-} (F) mice compared to wild-type mice (D). At E13.5, the size of geniculate ganglion in wild type mice (G) appeared larger compared to E11.5 (A), while in *Bdnf*^{-/-}/*Ntf4*^{-/-} (H) and *TrkB*^{-/-} mice (I) the ganglion continued to decrease in size. The inset in panel G represents a geniculate ganglion at higher magnification, illustrating a cell positively labeled for the neuronal marker TUJ-1, with a dark cytoplasm and a clear nucleus. Scale bar in I= 100 um and applies to A-I.

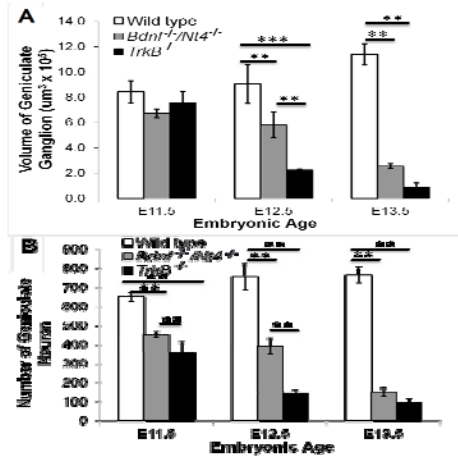


Figure 6. Geniculate ganglia from *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice decrease in volume and number of geniculate ganglion neurons between E11.5 and E13.5. (A) At E11.5, there was no difference between the geniculate ganglion volumes in wild type, *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice. At E12.5 the geniculate ganglion volume was reduced by 36% and 76% in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice, respectively, and it was significantly smaller in the *TrkB*^{-/-} mice compared to the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. At E13.5, there was a significant increase of geniculate ganglion volume in wild type mice compared to E11.5, while there were tremendous losses in *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice. There was no significant difference between the two mutant mice at this age. (B) At E11.5, the number of geniculate neurons was reduced by 31% and 45% in the *Bdnf*^{-/-}/*Ntf4*^{-/-} and *TrkB*^{-/-} mice compared to the wild type mice. At E12.5, the loss was about 48% in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and 81% in the *TrkB*^{-/-} mice compared to the wild type mice; there were significantly fewer neurons in the *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. At E13.5, the neuron number was reduced by 80% in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and 87% in *TrkB*^{-/-} mice compared to the wild-type mice, there was no significant differences between the two mutants. *p<0.05, **p<0.01 and ***p<0.001.

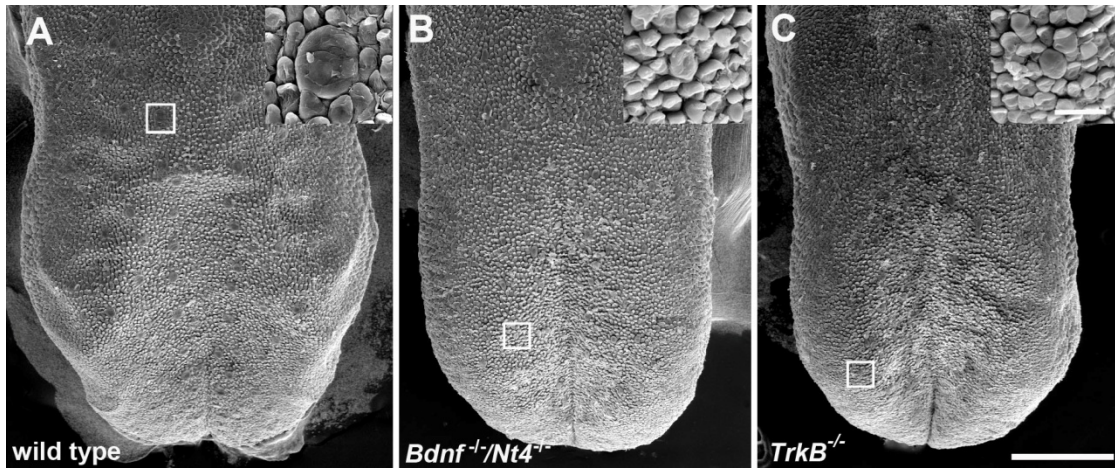


Figure 7. Both *Bdnf*^{-/-}/*Ntf4*^{-/-} mice *TrkB*^{-/-} mice have fewer and smaller fungiform papillae at P0.

Number and size of fungiform papillae were quantified with scanning electron microscopy (SEM) images of tongue from wild type mice (A), *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B) and *TrkB*^{-/-} mice (C). The inset in panel represents individual fungiform papillae from different genotypes. There were significantly fewer fungiform papillae at P0 in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (61±5) and *TrkB*^{-/-} mice (53±2) compared to wild-type mice (84±2, p<0.01), but there was no significant difference in the number and size of fungiform papillae between these two mutants. Scale bar in C=300 um and applies to A-C, scale bar in the inset panel =50 um and applies to three inset panels in A-C.

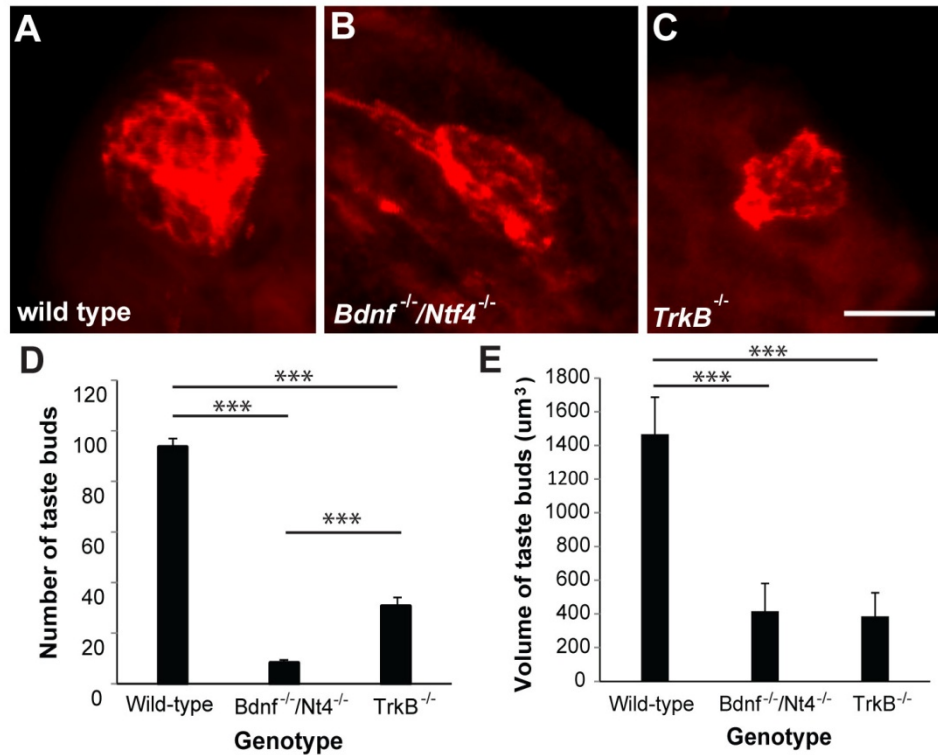


Figure 8. Comparison of taste bud number and size at P0.

Taste buds were visualized by anti-Troma1 staining from wild type mice (A), *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B) and *TrkB*^{-/-} mice (C). Taste bud number was reduced by 87% and 67% in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice, respectively; however, there were significantly more taste buds in the *TrkB*^{-/-} mice than the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (D). Taste bud volume was smaller in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice compared to the wild type mice, there was no significant difference between the two mutant groups (E). Scale bar in C= 10μm in C and applies to A-C. **p<0.01 and ***p<0.001.

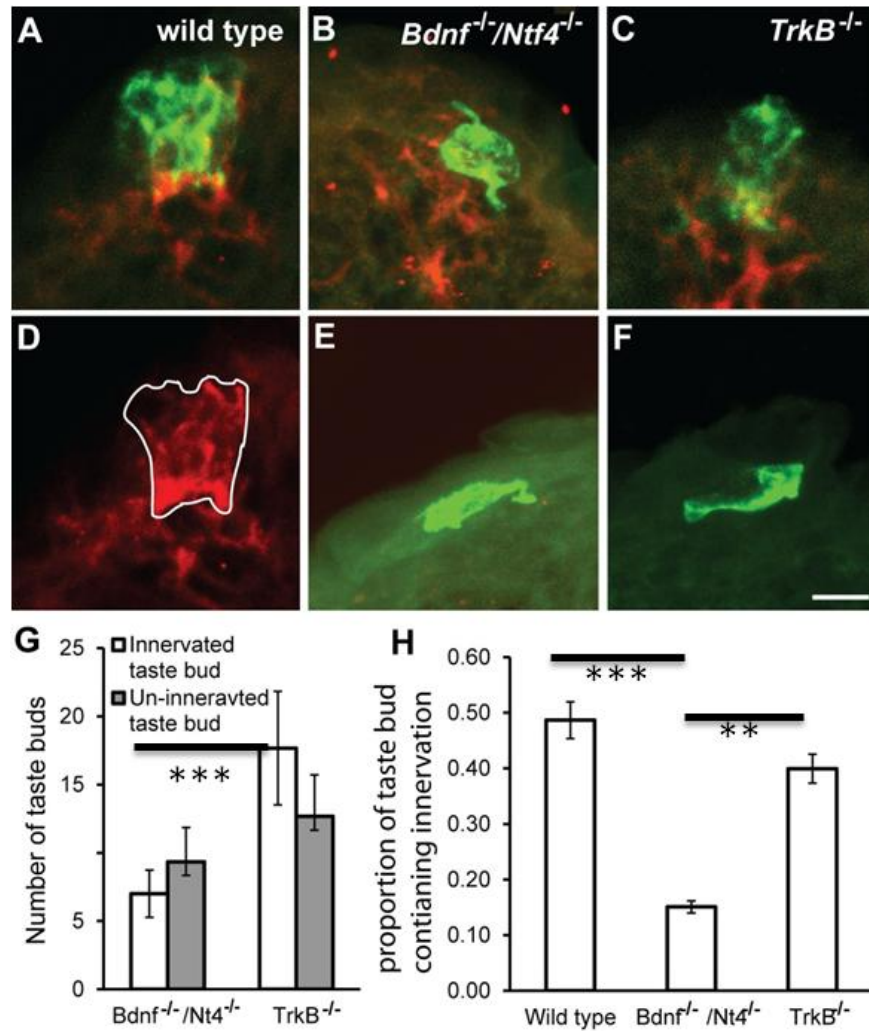


Figure 9. Comparison of taste bud innervations at P0.

Anti-Troma1 and anti-P2X3 were used to visualize taste bud and taste nerve in wild type mice (A), *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B) and *TrkB*^{-/-} mice (C). One optical section (D) shows area occupied by taste nerve within the taste bud. There were some un-innervated taste buds in the *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (E) and *TrkB*^{-/-} mice (F). (G) Comparison of number of innervated and un-innervated taste buds in *Bdnf*^{-/-}/*Ntf4*^{-/-} mice and *TrkB*^{-/-} mice. (H) Comparison of proportion of taste buds containing nerve fiber label. **p<0.01, *** p<0.001

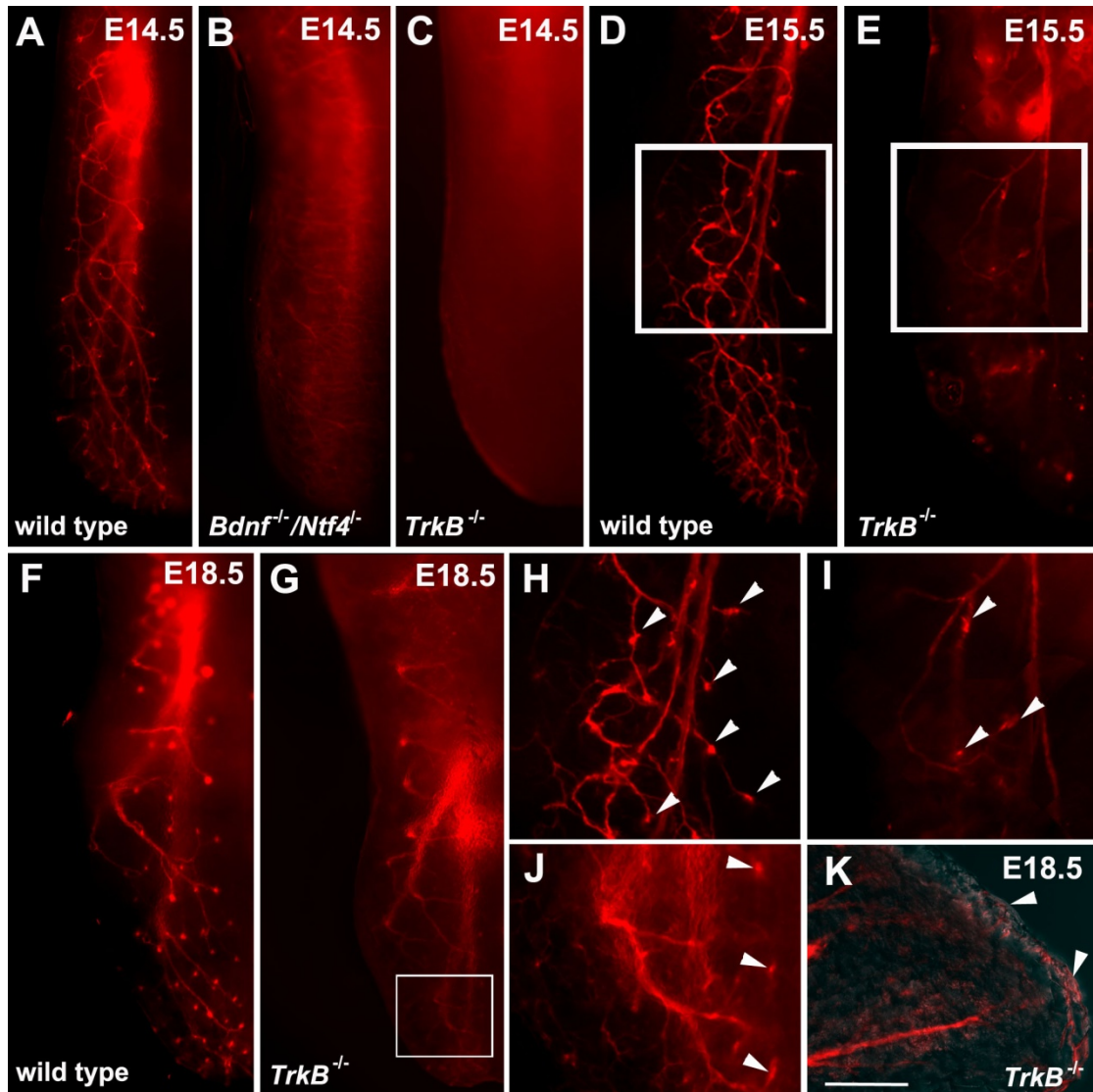


Figure 10. More innervation to the tongue surface remained in the *TrkB*^{-/-} mice than *Bdnf*^{-/-}/*Ntf4*^{-/-} mice. Dil-labeled half tongues from in wild type mice (A,D,F), *Bdnf*^{-/-}/*Ntf4*^{-/-} mice (B) and *TrkB*^{-/-} mice (C,E,G) at E14.5, E15.5 and E18.5. A higher magnification view of the E15.5 tongue (H,I, which corresponds to boxed area in D,E, respectively) and E18.5 (J, which corresponds to boxed area G). (K) A side view of innervation to the tip of tongue in *TrkB*^{-/-} mice.

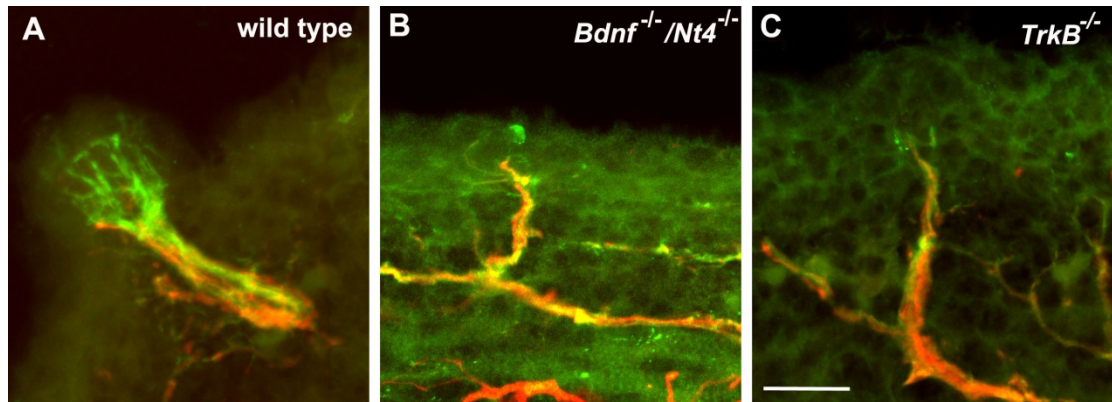


Figure 11. Comparison of taste fibers at E16.5.

Anti-P2X3 and anti-neurofilament antibodies were used to label taste nerve fibers in wild type mice (A), *Bdnf^{-/-}/Ntf4^{-/-}* mice (B) and *TrkB^{-/-}* mice (C). Scale bar in C= 20um and applies to A-C.

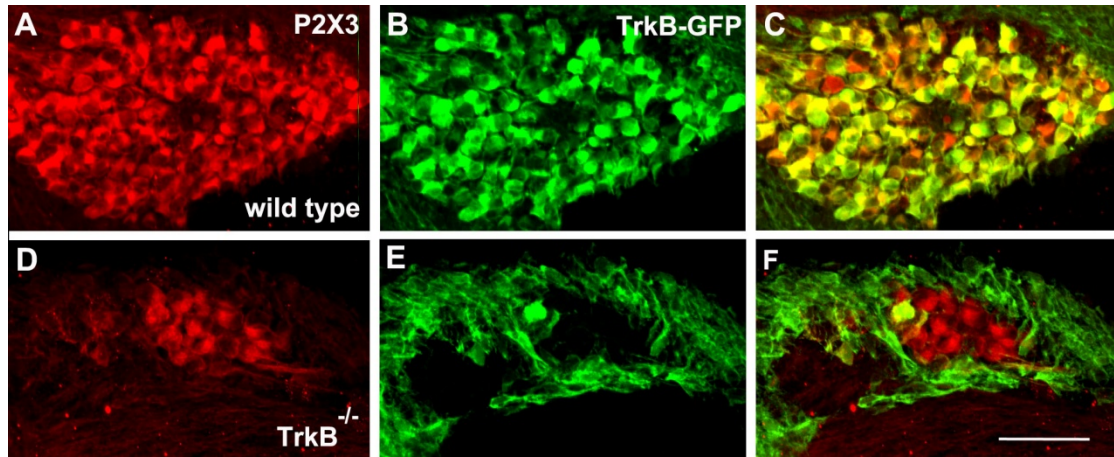


Figure 12. Remaining geniculate neurons *TrkB*^{-/-} mice do not express TrkB.

Anti-P2X3 was used to label all taste neurons in the geniculate (A,D), anti-GFP was used to label TrkB positive geniculate neurons (B,E). Merged pictures in (C,F). Scale bar = 50 um and applies to A-F.

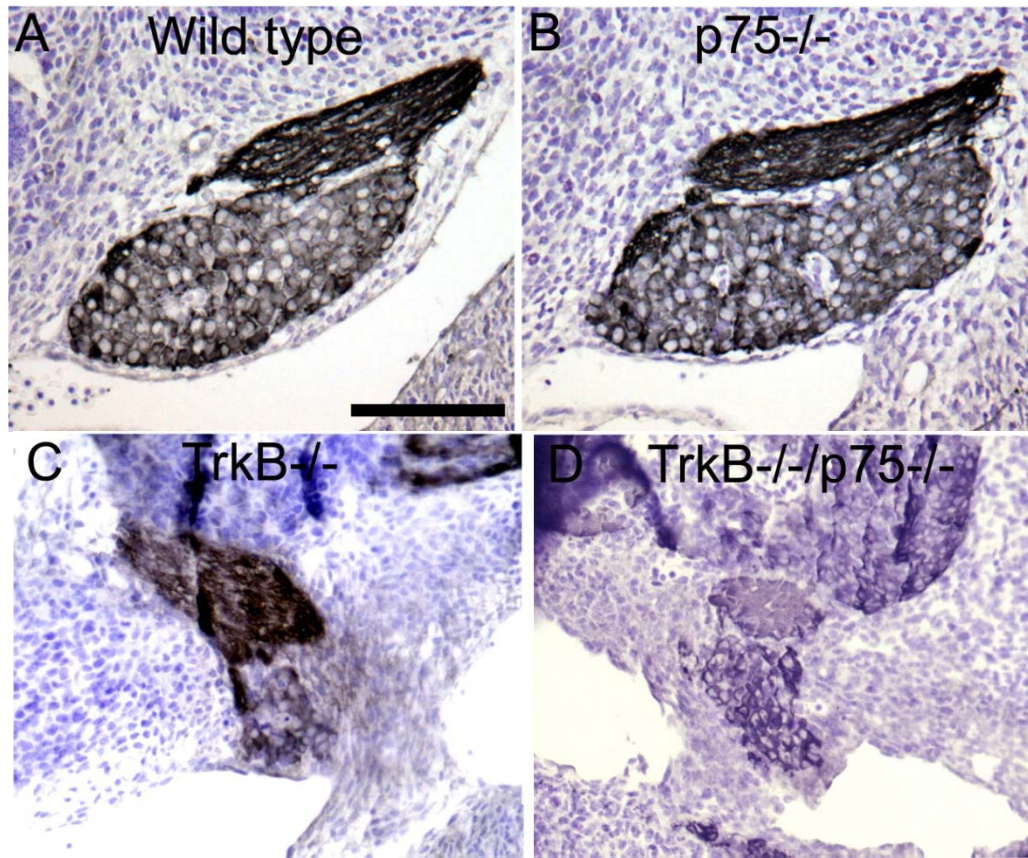


Figure 13. Tuj-1 labeling geniculate ganglion at E13.5.

Paraffin-embedded sections were stained with Tuj-1 antibody. Represented images of sectioned geniculate ganglion from wild type (A), *p75*^{-/-} (B), *TrkB*^{-/-} (C), and *TrkB*^{-/-}/*p75*^{-/-} (D) mice. Scale bar = 100um and applies to A-D.

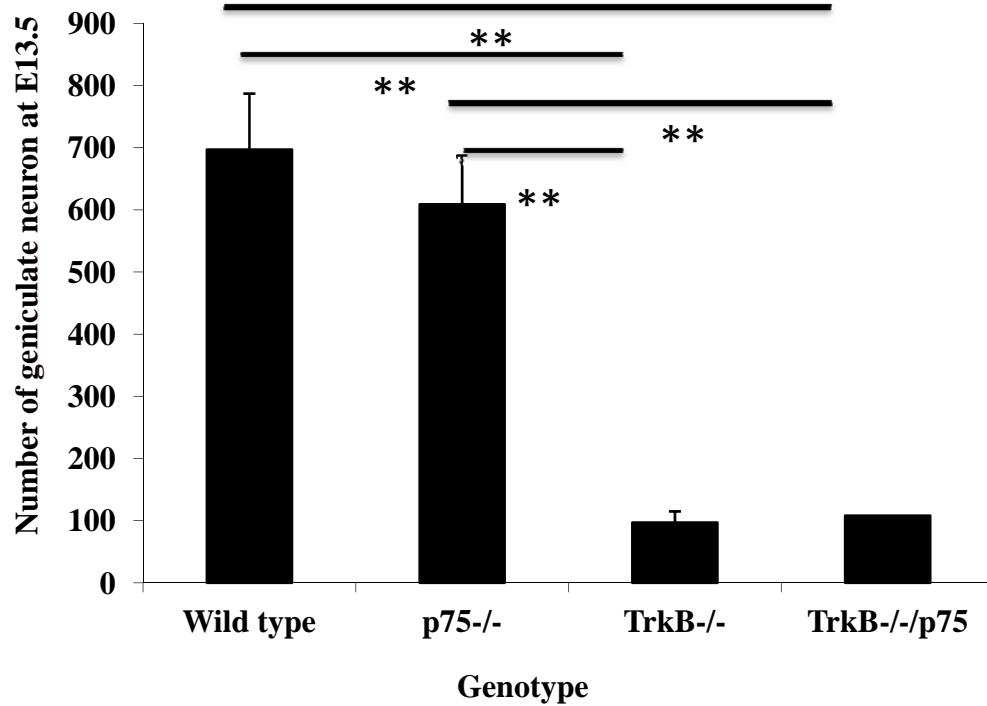


Figure 14. A comparison of the geniculate neuron number in wild type, *p75*^{-/-}, *TrkB*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice at E13.5.

Compared to wild type mice, there was no significant difference in neuron number in *p75*^{-/-} mice, while the geniculate neuron number decreased by 86% and 84% in *TrkB*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice, respectively. There is no significant difference between the *TrkB*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice.

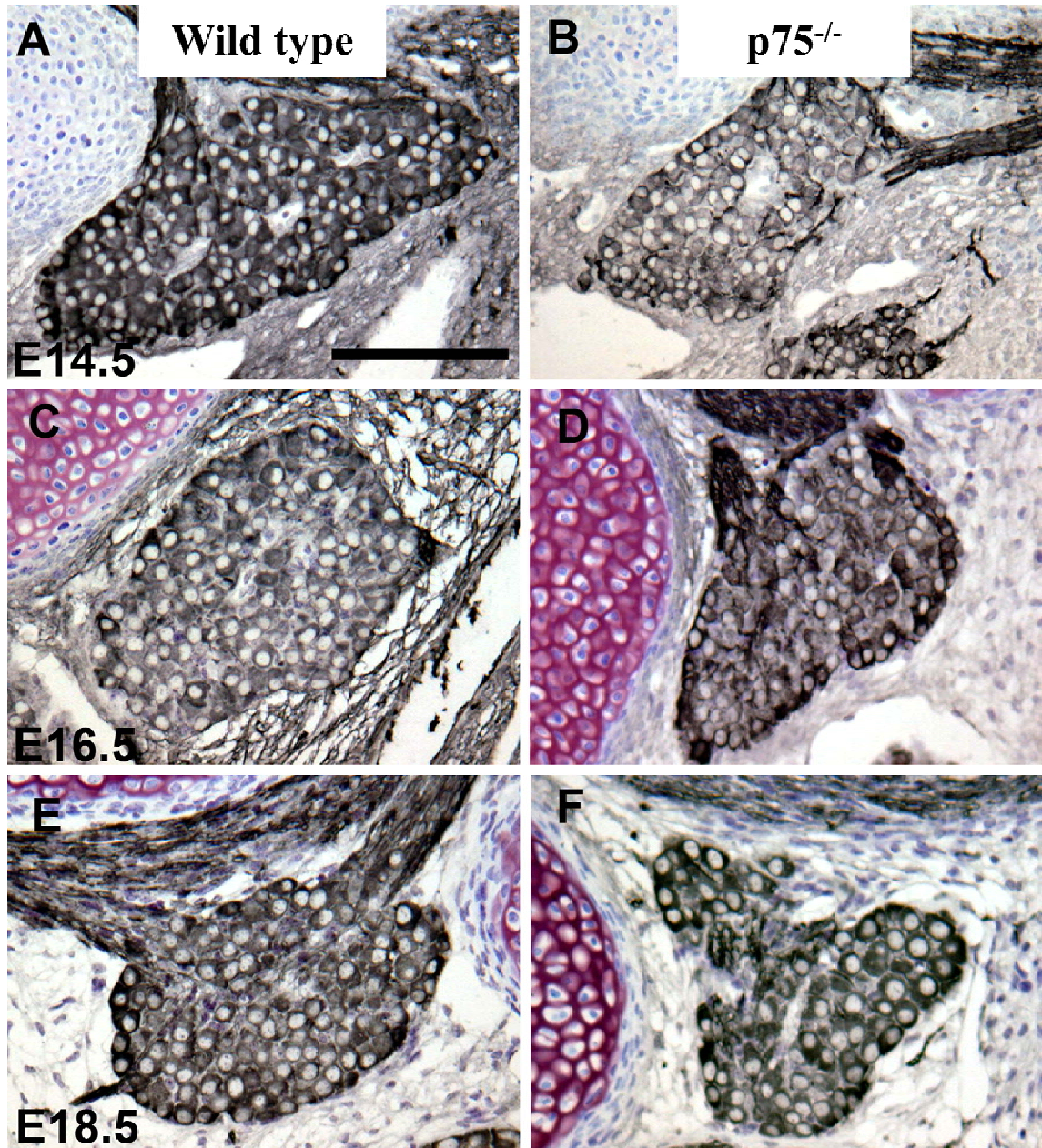


Figure 15. Tuj-1 labeling of the geniculate ganglion from E14.5 to E18.5.

Paraffin-embedded sections were stained with Tuj-1 antibody. Represented images of sectioned geniculate ganglion from wild type (A, C, E) and $p75^{-/-}$ (B, D, F) mice. Scale bar = 100um and applies to A-F.

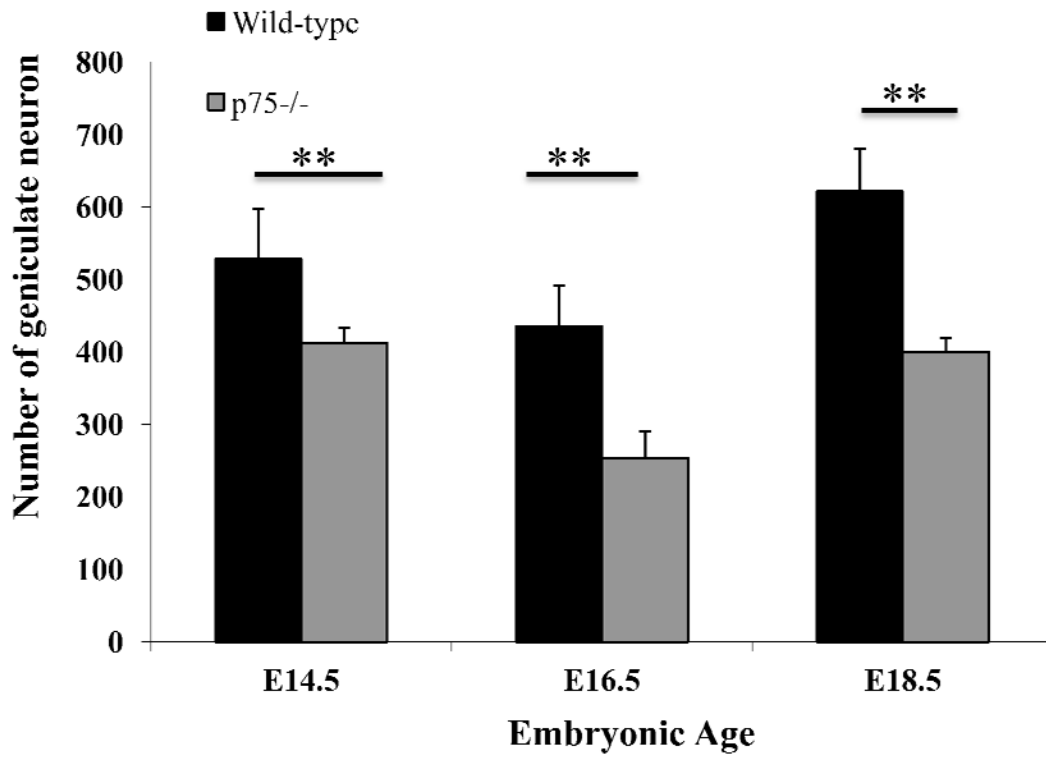


Figure 16. A comparison of the geniculate neuron number in wild type and *p75*^{-/-} mice from E14.5 to E18.5.

Compared to wild type mice, the geniculate neuron number in *p75*^{-/-} mice decreased by 22% (E14.5), 42% (E16.5) and 36% (E18.5), respectively.

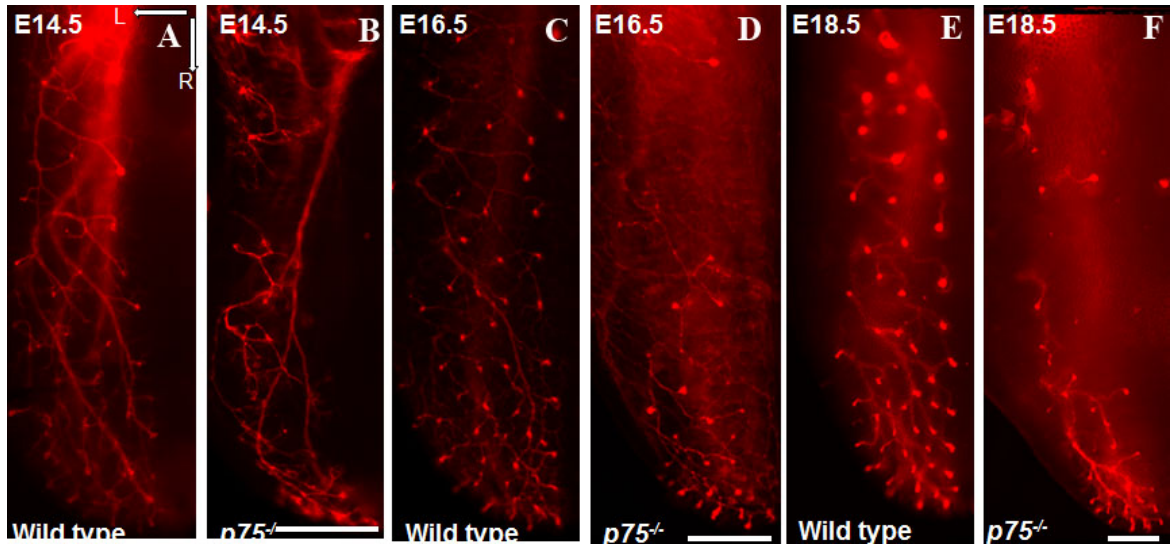


Figure 17. Disrupted innervation in $p75^{-/-}$ mice during development.

Dil-labeled half tongue in wild type (A,C,E) and $p75^{-/-}$ mice (B,D,F) from E14.5 to E18.5. Typical innervation pattern were seen in wild type mice through E14.5 to E18.5 (A, C, E). In the $p75^{-/-}$ mice, chorda tympani branching appears reduced and there are fewer specific locations where innervation appears to reach the tongue surface (neural buds). In particular, nerve branches appear to specifically avoid the tongue midline (B, D, F). Scale bar= 500 um, A-F.

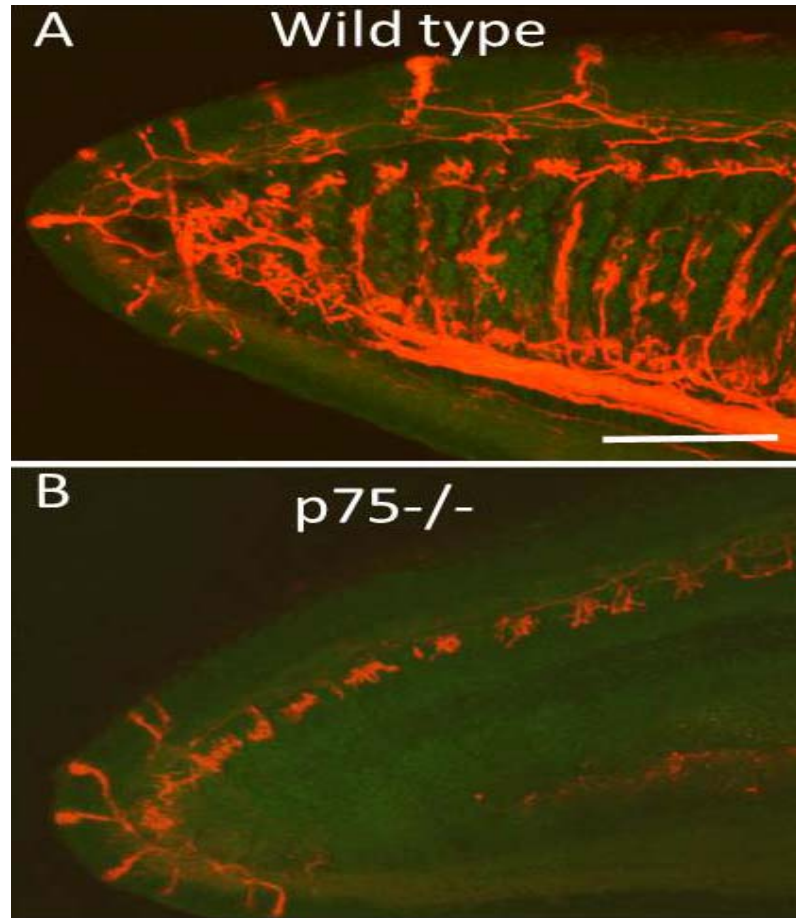


Figure 18. DiI-labeling of sagittal tongue sections.

At E18.5, chorda tympani fibers were present across the whole surface of the tongue in the wild type mice (A). In the $p75^{-/-}$ mice, fibers were only present at the tip of the tongue, while most of the chorda tympani fibers were missing from the posterior area of the tongue (B). Scale bar =500um, A-B.

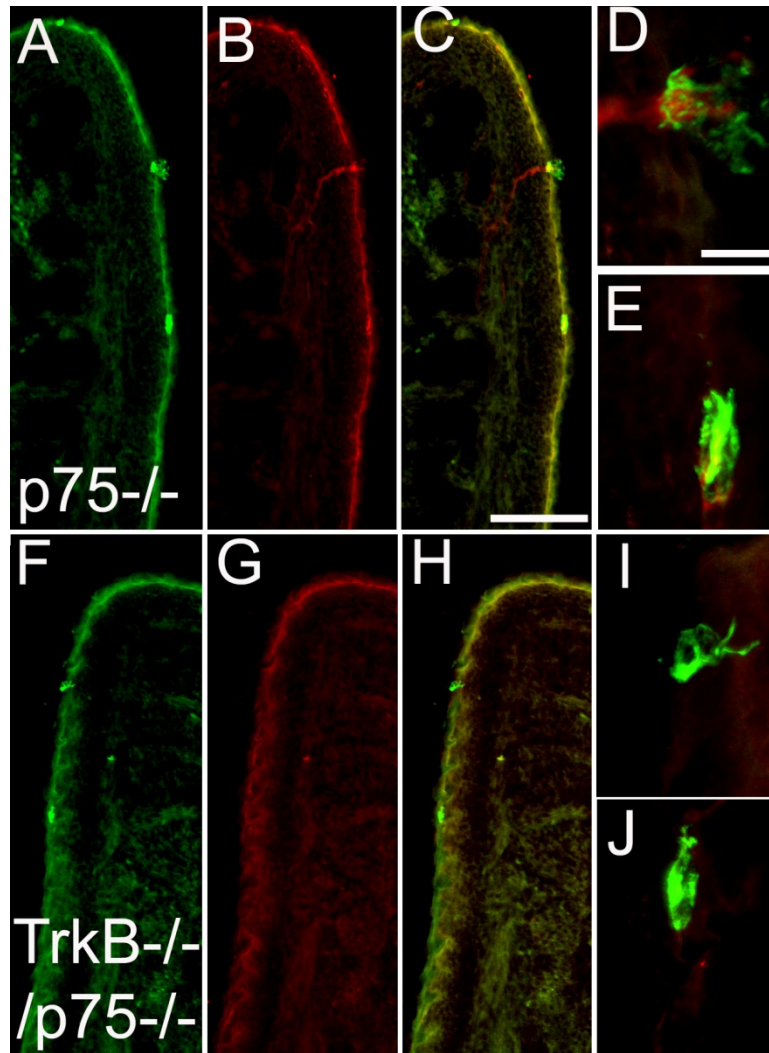


Figure 19. Troma1 and P2X3 staining of P0 tongue section.

Taste bud (Green, Troma1, A and F) and taste nerve (Red, P2X3, B and G) were visualized in a sagittal section of tongues from $p75^{-/-}$ (A-E) and $TrkB^{-/-}/p75^{-/-}$ mice (F-J). C and H are merged images and D, E, I, J are enlarged images from C and H. In the $p75^{-/-}$ mice, both innervated (D) and un-innervated (E) taste buds were present. In the $TrkB^{-/-}/p75^{-/-}$ mice, the remaining taste buds are un-innervated (I, J). Scale bar in C= 100um and applies A-C and F-H. Scale bar in D= 10um and applies to D, E, I, J.

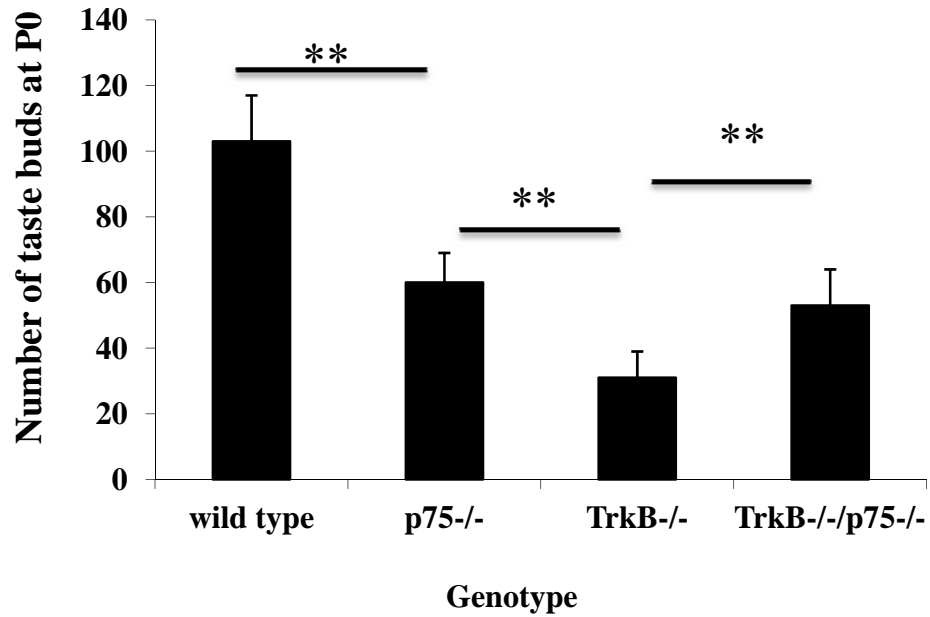


Figure 20. A comparison of taste bud number at P0.

Taste buds were visualized by Troma1 antibody and counted in serial sections. Compared to wild type mice, there was a significant decrease in taste bud number in *p75*^{-/-}, *TrkB*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice. The decrease was 42%, 70% and 49%, respectively. *TrkB*^{-/-} mice has significantly less taste buds compared to *p75*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice. There was no significant difference between *p75*^{-/-} and *TrkB*^{-/-}/*p75*^{-/-} mice.

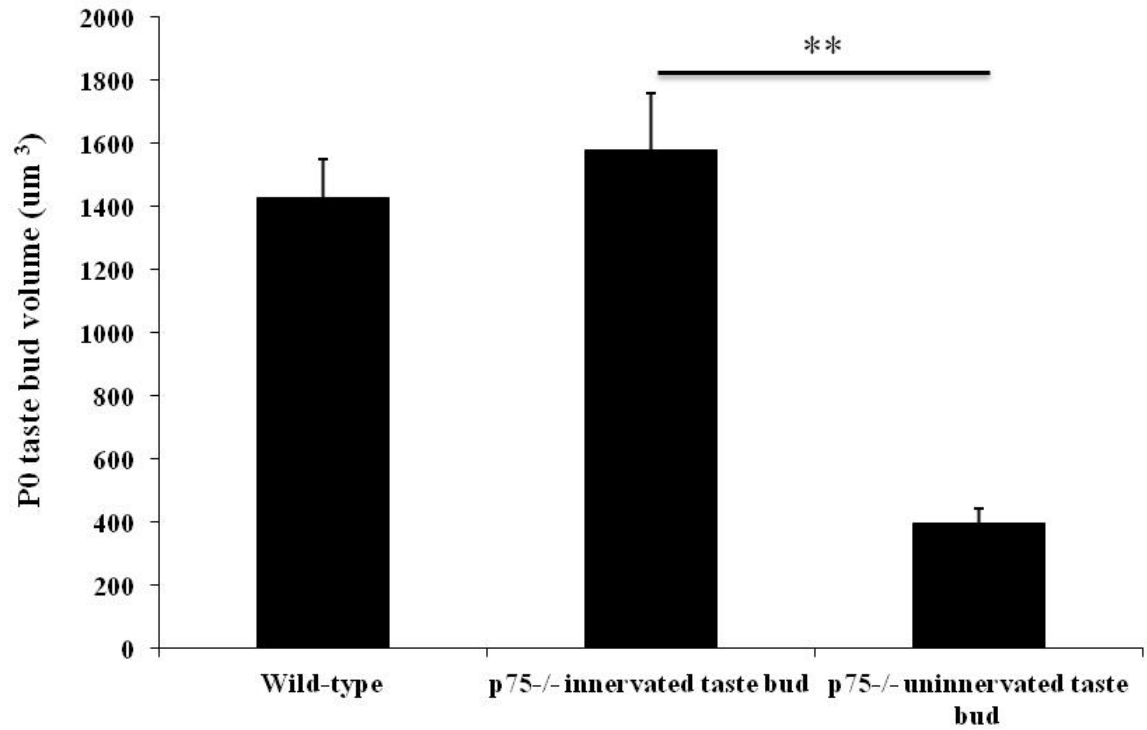


Figure 21. A comparison of taste bud volume at P0.

Taste bud volume was measured in wild type mice and *p75^{-/-}* mice. Compared to the wild type mice, innervated taste buds in *p75^{-/-}* mice had similar size while un-innervated taste buds were significantly smaller.

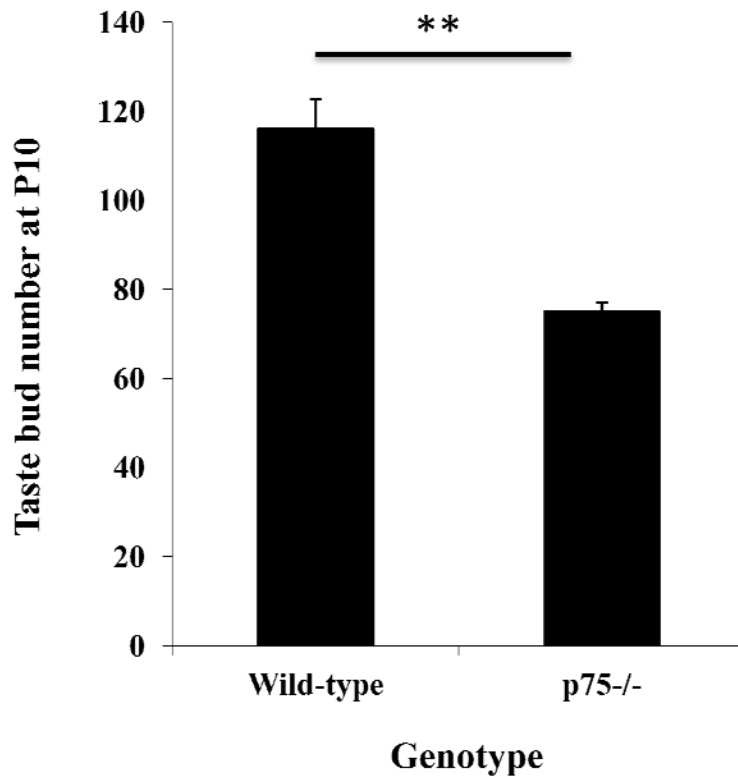


Figure 22. Taste bud number in *p75^{-/-}* mice at P10.

Compared to wild type mice, the number of taste buds in *p75^{-/-}* mice was decreased by 35%. Compared to the taste bud number loss in *p75^{-/-}* mice at P0, there was no further loss of taste bud number in *p75^{-/-}* mice at this age.

REFERENCES

1. Farbman AI (1965) Electron Microscope Study of the Developing Taste Bud in Rat Fungiform Papilla. *Dev Biol* 11: 110-135.
2. Delay RJ, Kinnamon JC, Roper SD (1986) Ultrastructure of mouse vallate taste buds: II. Cell types and cell lineage. *J Comp Neurol* 253: 242-252.
3. Witt M, Reutter K (1997) Scanning electron microscopical studies of developing gustatory papillae in humans. *Chem Senses* 22: 601-612.
4. Pumplun DW, Yu C, Smith DV (1997) Light and dark cells of rat vallate taste buds are morphologically distinct cell types. *J Comp Neurol* 378: 389-410.
5. Northcutt RG (2004) Taste buds: development and evolution. *Brain Behav Evol* 64: 198-206.
6. Yee CL, Yang R, Bottger B, Finger TE, Kinnamon JC (2001) "Type III" cells of rat taste buds: immunohistochemical and ultrastructural studies of neuron-specific enolase, protein gene product 9.5, and serotonin. *J Comp Neurol* 440: 97-108.
7. Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, et al. (2001) Mammalian sweet taste receptors. *Cell* 106: 381-390.
8. Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, et al. (2003) The receptors for mammalian sweet and umami taste. *Cell* 115: 255-266.
9. Li X (2009) T1R receptors mediate mammalian sweet and umami taste. *Am J Clin Nutr* 90: 733S-737S.
10. Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, et al. (2002) An amino-acid taste receptor. *Nature* 416: 199-202.
11. Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, et al. (2000) T2Rs function as bitter taste receptors. *Cell* 100: 703-711.
12. Montmayeur JP, Matsunami H (2002) Receptors for bitter and sweet taste. *Curr Opin Neurobiol* 12: 366-371.

13. LopezJimenez ND, Cavenagh MM, Sainz E, Cruz-Ithier MA, Battey JF, et al. (2006) Two members of the TRPP family of ion channels, Pkd1l3 and Pkd2l1, are co-expressed in a subset of taste receptor cells. *J Neurochem* 98: 68-77.
14. Ishimaru Y, Inada H, Kubota M, Zhuang H, Tominaga M, et al. (2006) Transient receptor potential family members PKD1L3 and PKD2L1 form a candidate sour taste receptor. *Proc Natl Acad Sci U S A* 103: 12569-12574.
15. Huang AL, Chen X, Hoon MA, Chandrashekar J, Guo W, et al. (2006) The cells and logic for mammalian sour taste detection. *Nature* 442: 934-938.
16. Stevens DR, Seifert R, Bufe B, Muller F, Kremmer E, et al. (2001) Hyperpolarization-activated channels HCN1 and HCN4 mediate responses to sour stimuli. *Nature* 413: 631-635.
17. Ugawa S, Minami Y, Guo W, Saishin Y, Takatsuji K, et al. (1998) Receptor that leaves a sour taste in the mouth. *Nature* 395: 555-556.
18. Lin W, Burks CA, Hansen DR, Kinnamon SC, Gilbertson TA (2004) Taste receptor cells express pH-sensitive leak K⁺ channels. *J Neurophysiol* 92: 2909-2919.
19. Richter TA, Dvoryanchikov GA, Chaudhari N, Roper SD (2004) Acid-sensitive two-pore domain potassium (K2P) channels in mouse taste buds. *J Neurophysiol* 92: 1928-1936.
20. Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M (1997) A proton-gated cation channel involved in acid-sensing. *Nature* 386: 173-177.
21. Lyall V, Alam RI, Malik SA, Phan TH, Vinnikova AK, et al. (2004) Basolateral Na⁺-H⁺ exchanger-1 in rat taste receptor cells is involved in neural adaptation to acidic stimuli. *J Physiol* 556: 159-173.
22. Cummings TA, Kinnamon SC (1992) Apical K⁺ channels in *Necturus* taste cells. Modulation by intracellular factors and taste stimuli. *J Gen Physiol* 99: 591-613.
23. Heck GL, Mierson S, DeSimone JA (1984) Salt taste transduction occurs through an amiloride-sensitive sodium transport pathway. *Science* 223: 403-405.
24. Levi-Montalcini R (1987) The nerve growth factor: thirty-five years later. *EMBO J* 6: 1145-1154.
25. Farinas I, Wilkinson GA, Backus C, Reichardt LF, Patapoutian A (1998) Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo. *Neuron* 21: 325-334.
26. Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24: 677-736.

27. Huang EJ, Reichardt LF (2003) TRK Receptors: Roles in Neuronal Signal Transduction. *Annu Rev Biochem*.
28. Kaplan DR, Miller FD (1997) Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol* 9: 213-221.
29. Luikart BW, Nef S, Shipman T, Parada LF (2003) In vivo role of truncated trkb receptors during sensory ganglion neurogenesis. *Neuroscience* 117: 847-858.
30. Frade JM, Rodriguez-Tebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* 383: 166-168.
31. Lee KF, Li E, Huber LJ, Landis SC, Sharpe AH, et al. (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* 69: 737-749.
32. Casaccia-Bonnel P, Gu C, Khursigara G, Chao MV (1999) p75 neurotrophin receptor as a modulator of survival and death decisions. *Microsc Res Tech* 45: 217-224.
33. Baker CV, Bronner-Fraser M (2001) Vertebrate cranial placodes I. Embryonic induction. *Dev Biol* 232: 1-61.
34. Gross JB, Gottlieb AA, Barlow LA (2003) Gustatory neurons derived from epibranchial placodes are attracted to, and trophically supported by, taste bud-bearing endoderm in vitro. *Dev Biol* 264: 467-481.
35. Harlow DE, Barlow LA (2007) Embryonic origin of gustatory cranial sensory neurons. *Dev Biol* 310: 317-328.
36. Begbie J, Graham A (2001) Integration between the epibranchial placodes and the hindbrain. *Science* 294: 595-598.
37. Begbie J, Brunet JF, Rubenstein JL, Graham A (1999) Induction of the epibranchial placodes. *Development* 126: 895-902.
38. Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, et al. (1998) The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* 20: 483-494.
39. Morin X, Cremer H, Hirsch MR, Kapur RP, Goridis C, et al. (1997) Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. *Neuron* 18: 411-423.
40. Dauger S, Pattyn A, Lofaso F, Gaultier C, Goridis C, et al. (2003) Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways. *Development* 130: 6635-6642.

41. Altman J, Bayer SA (1982) Development of the cranial nerve ganglia and related nuclei in the rat. *Adv Anat Embryol Cell Biol* 74: 1-90.
42. Lopez GF, Krimm RF (2006) Refinement of innervation accuracy following initial targeting of peripheral gustatory fibers. *J Neurobiol* 66: 1033-1043.
43. Mbiene JP (2004) Taste placodes are primary targets of geniculate but not trigeminal sensory axons in mouse developing tongue. *J Neurocytol* 33: 617-629.
44. Farbman AI, Mbiene JP (1991) Early development and innervation of taste bud-bearing papillae on the rat tongue. *J Comp Neurol* 304: 172-186.
45. Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, et al. (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* 375: 235-238.
46. Liu X, Ernfors P, Wu H, Jaenisch R (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature* 375: 238-241.
47. Liebl DJ, Tessarollo L, Palko ME, Parada LF (1997) Absence of sensory neurons before target innervation in brain-derived neurotrophic factor-, neurotrophin 3-, and TrkC-deficient embryonic mice. *J Neurosci* 17: 9113-9121.
48. Erickson JT, Conover JC, Borday V, Champagnat J, Barbacid M, et al. (1996) Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J Neurosci* 16: 5361-5371.
49. Ringstedt T, Ibanez CF, Nosrat CA (1999) Role of brain-derived neurotrophic factor in target invasion in the gustatory system. *J Neurosci* 19: 3507-3518.
50. Krimm RF, Miller KK, Kitzman PH, Davis BM, Albers KM (2001) Epithelial overexpression of BDNF or NT4 disrupts targeting of taste neurons that innervate the anterior tongue. *Dev Biol* 232: 508-521.
51. Patel AV, Krimm RF (2012) Neurotrophin-4 regulates the survival of gustatory neurons earlier in development using a different mechanism than brain-derived neurotrophic factor. *Dev Biol* 365: 50-60.
52. Patel AV, Krimm RF (2010) BDNF is required for the survival of differentiated geniculate ganglion neurons. *Dev Biol* 340: 419-429.
53. Mbiene JP, Mistretta CM (1997) Initial innervation of embryonic rat tongue and developing taste papillae: nerves follow distinctive and spatially restricted pathways. *Acta Anat (Basel)* 160: 139-158.

54. Rochlin MW, O'Connor R, Giger RJ, Verhaagen J, Farbman AI (2000) Comparison of neurotrophin and repellent sensitivities of early embryonic geniculate and trigeminal axons. *J Comp Neurol* 422: 579-593.
55. Runge EM, Hoshino N, Biehl MJ, Ton S, Rochlin MW (2012) Neurotrophin-4 is more potent than brain-derived neurotrophic factor in promoting, attracting and suppressing geniculate ganglion neurite outgrowth. *Dev Neurosci* 34: 389-401.
56. Ma L, Lopez GF, Krimm RF (2009) Epithelial-derived brain-derived neurotrophic factor is required for gustatory neuron targeting during a critical developmental period. *J Neurosci* 29: 3354-3364.
57. Yu TW, Bargmann CI (2001) Dynamic regulation of axon guidance. *Nat Neurosci* 4 Suppl: 1169-1176.
58. Wilkinson DG (2001) Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* 2: 155-164.
59. Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC (1996) Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* 383: 525-528.
60. Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78: 425-435.
61. Kramer SG, Kidd T, Simpson JH, Goodman CS (2001) Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. *Science* 292: 737-740.
62. Giger RJ, Wolfer DP, De Wit GM, Verhaagen J (1996) Anatomy of rat semaphorin III/collapsin-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. *J Comp Neurol* 375: 378-392.
63. Vilbig R, Cosmano J, Giger R, Rochlin MW (2004) Distinct roles for Sema3A, Sema3F, and an unidentified trophic factor in controlling the advance of geniculate axons to gustatory lingual epithelium. *J Neurocytol* 33: 591-606.
64. Dillon TE, Saldanha J, Giger R, Verhaagen J, Rochlin MW (2004) Sema3A regulates the timing of target contact by cranial sensory axons. *J Comp Neurol* 470: 13-24.
65. Lopez GF, Krimm RF (2006) Epithelial overexpression of BDNF and NT4 produces distinct gustatory axon morphologies that disrupt initial targeting. *Dev Biol* 292: 457-468.
66. Dontchev VD, Letourneau PC (2002) Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. *J Neurosci* 22: 6659-6669.

67. Mistretta CM, Liu HX, Gaffield W, MacCallum DK (2003) Cyclopamine and jervine in embryonic rat tongue cultures demonstrate a role for Shh signaling in taste papilla development and patterning: fungiform papillae double in number and form in novel locations in dorsal lingual epithelium. *Dev Biol* 254: 1-18.
68. Zhou Y, Liu HX, Mistretta CM (2006) Bone morphogenetic proteins and noggin: inhibiting and inducing fungiform taste papilla development. *Dev Biol* 297: 198-213.
69. Liu F, Thirumangalathu S, Gallant NM, Yang SH, Stoick-Cooper CL, et al. (2007) Wnt-beta-catenin signaling initiates taste papilla development. *Nat Genet* 39: 106-112.
70. Iwatsuki K, Liu HX, Gronder A, Singer MA, Lane TF, et al. (2007) Wnt signaling interacts with Shh to regulate taste papilla development. *Proc Natl Acad Sci U S A* 104: 2253-2258.
71. Mbiene JP, Maccallum DK, Mistretta CM (1997) Organ cultures of embryonic rat tongue support tongue and gustatory papilla morphogenesis in vitro without intact sensory ganglia. *J Comp Neurol* 377: 324-340.
72. Miller IJ, Jr., Preslar AJ (1975) Spatial distribution of rat fungiform papillae. *Anat Rec* 181: 679-684.
73. Nosrat CA, Olson L (1995) Brain-derived neurotrophic factor mRNA is expressed in the developing taste bud-bearing tongue papillae of rat. *J Comp Neurol* 360: 698-704.
74. Nosrat CA, Ebendal T, Olson L (1996) Differential expression of brain-derived neurotrophic factor and neurotrophin 3 mRNA in lingual papillae and taste buds indicates roles in gustatory and somatosensory innervation. *J Comp Neurol* 376: 587-602.
75. Nosrat CA, MacCallum DK, Mistretta CM (2001) Distinctive spatiotemporal expression patterns for neurotrophins develop in gustatory papillae and lingual tissues in embryonic tongue organ cultures. *Cell Tissue Res* 303: 35-45.
76. Krimm RF, Davis BM, Noel T, Albers KM (2006) Overexpression of neurotrophin 4 in skin enhances myelinated sensory endings but does not influence sensory neuron number. *J Comp Neurol* 498: 455-465.
77. Hosley MA, Hughes SE, Morton LL, Oakley B (1987) A sensitive period for the neural induction of taste buds. *J Neurosci* 7: 2075-2080.
78. Whitehead MC, Kachele DL (1994) Development of fungiform papillae, taste buds, and their innervation in the hamster. *J Comp Neurol* 340: 515-530.
79. Barlow LA (2001) Specification of pharyngeal endoderm is dependent on early signals from axial mesoderm. *Development* 128: 4573-4583.

80. Barlow LA, Northcutt RG (1995) Embryonic origin of amphibian taste buds. *Dev Biol* 169: 273-285.
81. Brockes JP (1997) Amphibian limb regeneration: rebuilding a complex structure. *Science* 276: 81-87.
82. Barlow LA, Chien CB, Northcutt RG (1996) Embryonic taste buds develop in the absence of innervation. *Development* 122: 1103-1111.
83. Fritzscht B, Sarai PA, Barbacid M, Silos-Santiago I (1997) Mice with a targeted disruption of the neurotrophin receptor *trkB* lose their gustatory ganglion cells early but do develop taste buds. *Int J Dev Neurosci* 15: 563-576.
84. Mistretta CM, Hill DL (1995) Development of the taste system: basic neurobiology. In: R.L. D, editor. *Handbook of Olfaction and Gustation*. New York: Marcel Dekker, pp. 635-668.
85. Liebl DJ, Mbiene JP, Parada LF (1999) NT4/5 mutant mice have deficiency in gustatory papillae and taste bud formation. *Dev Biol* 213: 378-389.
86. Mistretta CM, Goosens KA, Farinas I, Reichardt LF (1999) Alterations in size, number, and morphology of gustatory papillae and taste buds in BDNF null mutant mice demonstrate neural dependence of developing taste organs. *J Comp Neurol* 409: 13-24.
87. Nosrat CA, Blomlof J, ElShamy WM, Ernfors P, Olson L (1997) Lingual deficits in BDNF and NT3 mutant mice leading to gustatory and somatosensory disturbances, respectively. *Development* 124: 1333-1342.
88. Sun H, Oakley B (2002) Development of anterior gustatory epithelia in the palate and tongue requires epidermal growth factor receptor. *Dev Biol* 242: 31-43.
89. Patel AV, Huang T, Krimm RF (2010) Lingual and palatal gustatory afferents each depend on both BDNF and NT-4, but the dependence is greater for lingual than palatal afferents. *J Comp Neurol* 518: 3290-3301.
90. Nosrat IV, Margolskee RF, Nosrat CA (2012) Targeted taste cell-specific overexpression of brain-derived neurotrophic factor in adult taste buds elevates phosphorylated TrkB protein levels in taste cells, increases taste bud size, and promotes gustatory innervation. *J Biol Chem* 287: 16791-16800.
91. Hoshino N, Vatterott P, Egwiekhor A, Rochlin MW (2010) Brain-derived neurotrophic factor attracts geniculate ganglion neurites during embryonic targeting. *Dev Neurosci* 32: 184-196.
92. Oakley B, Brandemihl A, Cooper D, Lau D, Lawton A, et al. (1998) The morphogenesis of mouse vallate gustatory epithelium and taste buds requires BDNF-dependent taste neurons. *Brain Res Dev Brain Res* 105: 85-96.

93. Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* 4: 299-309.
94. Ito A, Nosrat CA (2009) Gustatory papillae and taste bud development and maintenance in the absence of TrkB ligands BDNF and NT-4. *Cell Tissue Res* 337: 349-359.
95. Li L, Rutlin M, Abaira VE, Cassidy C, Kus L, et al. (2011) The functional organization of cutaneous low-threshold mechanosensory neurons. *Cell* 147: 1615-1627.
96. Hosley MA, Hughes SE, Oakley B (1987) Neural induction of taste buds. *J Comp Neurol* 260: 224-232.
97. Hosley MA, Oakley B (1987) Postnatal development of the vallate papilla and taste buds in rats. *Anat Rec* 218: 216-222.
98. Oakley B, Witt M (2004) Building sensory receptors on the tongue. *J Neurocytol* 33: 631-646.
99. Finger TE, Danilova V, Barrows J, Bartel DL, Vigers AJ, et al. (2005) ATP signaling is crucial for communication from taste buds to gustatory nerves. *Science* 310: 1495-1499.
100. Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, et al. (1993) Targeted disruption of the *trkB* neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* 75: 113-122.
101. Ninkina N, Adu J, Fischer A, Pinon LG, Buchman VL, et al. (1996) Expression and function of TrkB variants in developing sensory neurons. *Embo J* 15: 6385-6393.
102. Escandon E, Soppet D, Rosenthal A, Mendoza-Ramirez JL, Szonyi E, et al. (1994) Regulation of neurotrophin receptor expression during embryonic and postnatal development. *J Neurosci* 14: 2054-2068.
103. Farinas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* 369: 658-661.
104. Davies AM (1994) Neurobiology. Tracking neurotrophin function. *Nature* 368: 193-194.
105. Buchman VL, Davies AM (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development* 118: 989-1001.
106. ElShamy WM, Ernfors P (1997) Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 complement and cooperate with each other sequentially during visceral neuron development. *Journal of Neuroscience* 17: 8667-8675.
107. Nosrat IV, Agerman K, Marinescu A, Ernfors P, Nosrat CA (2004) Lingual deficits in neurotrophin double knockout mice. *J Neurocytol* 33: 607-615.

108. Lee FS, Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A* 98: 3555-3560.
109. Assaife-Lopes N, Sousa VC, Pereira DB, Ribeiro JA, Chao MV, et al. (2010) Activation of adenosine A2A receptors induces TrkB translocation and increases BDNF-mediated phospho-TrkB localization in lipid rafts: implications for neuromodulation. *J Neurosci* 30: 8468-8480.
110. Wiese S, Jablonka S, Holtmann B, Orel N, Rajagopal R, et al. (2007) Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. *Proc Natl Acad Sci U S A* 104: 17210-17215.
111. Dando R, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD (2012) Adenosine enhances sweet taste through A2B receptors in the taste bud. *J Neurosci* 32: 322-330.
112. Kataoka S, Baquero A, Yang D, Shultz N, Vandenbeuch A, et al. (2012) A2BR adenosine receptor modulates sweet taste in circumvallate taste buds. *PLoS One* 7: e30032.
113. Kaelin-Lang A, Lauterburg T, Burgunder JM (1998) Expression of adenosine A2a receptor gene in rat dorsal root and autonomic ganglia. *Neurosci Lett* 246: 21-24.
114. Nagato T, Matsumoto K, Tanioka H, Kodama J, Toh H (1995) Effect of denervation on morphogenesis of the rat fungiform papilla. *Acta Anat (Basel)* 153: 301-309.
115. Sollars SI, Smith PC, Hill DL (2002) Time course of morphological alterations of fungiform papillae and taste buds following chorda tympani transection in neonatal rats. *J Neurobiol* 51: 223-236.
116. Sollars SI (2005) Chorda tympani nerve transection at different developmental ages produces differential effects on taste bud volume and papillae morphology in the rat. *J Neurobiol* 64: 310-320.
117. Guagliardo NA, Hill DL (2007) Fungiform taste bud degeneration in C57BL/6J mice following chorda-lingual nerve transection. *J Comp Neurol* 504: 206-216.
118. Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, et al. (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature* 426: 74-78.
119. Ng BK, Chen L, Mandemakers W, Cosgaya JM, Chan JR (2007) Anterograde transport and secretion of brain-derived neurotrophic factor along sensory axons promote Schwann cell myelination. *J Neurosci* 27: 7597-7603.
120. Nave KA (2010) Myelination and support of axonal integrity by glia. *Nature* 468: 244-252.
121. Zaidi FN, Whitehead MC (2006) Discrete innervation of murine taste buds by peripheral taste neurons. *J Neurosci* 26: 8243-8253.

122. Cho TT, Farbman AI (1999) Neurotrophin receptors in the geniculate ganglion. *Brain Res Mol Brain Res* 68: 1-13.
123. Farbman AI, Brann JH, Rozenblat A, Rochlin MW, Weiler E, et al. (2004) Developmental expression of neurotrophin receptor genes in rat geniculate ganglion neurons. *J Neurocytol* 33: 331-343.
124. D'Autreaux F, Coppola E, Hirsch MR, Birchmeier C, Brunet JF (2011) Homeoprotein Phox2b commands a somatic-to-visceral switch in cranial sensory pathways. *Proc Natl Acad Sci U S A* 108: 20018-20023.
125. Matsumoto I, Emori Y, Ninomiya Y, Abe K (2001) A comparative study of three cranial sensory ganglia projecting into the oral cavity: in situ hybridization analyses of neurotrophin receptors and thermosensitive cation channels. *Brain Res Mol Brain Res* 93: 105-112.
126. Stucky CL, DeChiara T, Lindsay RM, Yancopoulos GD, Koltzenburg M (1998) Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. *J Neurosci* 18: 7040-7046.
127. Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* 13: 203-215.
128. Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *J Biol Chem* 269: 6884-6891.
129. Davies AM, Lee KF, Jaenisch R (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* 11: 565-574.
130. Lee KF, Bachman K, Landis S, Jaenisch R (1994) Dependence on p75 for innervation of some sympathetic targets. *Science* 263: 1447-1449.
131. Bamji SX, Majdan M, Poznaniak CD, Belliveau DJ, Aloyz R, et al. (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J Cell Biol* 140: 911-923.
132. Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, et al. (1997) Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. *J Neurosci* 17: 7594-7605.
133. Van der Zee CE, Ross GM, Riopelle RJ, Hagg T (1996) Survival of cholinergic forebrain neurons in developing p75NGFR-deficient mice. *Science* 274: 1729-1732.
134. Casaccia-Bonnel P, Carter BD, Dobrowsky RT, Chao MV (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* 383: 716-719.

135. Davey F, Davies AM (1998) TrkB signalling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. *Curr Biol* 8: 915-918.
136. Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, et al. (1993) Induction of apoptosis by the low-affinity NGF receptor. *Science* 261: 345-348.
137. Barrett GL, Bartlett PF (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc Natl Acad Sci U S A* 91: 6501-6505.
138. Feinstein E, Kimchi A, Wallach D, Boldin M, Varfolomeev E (1995) The death domain: a module shared by proteins with diverse cellular functions. *Trends Biochem Sci* 20: 342-344.
139. Hofmann K, Tschopp J (1995) The death domain motif found in Fas (Apo-1) and TNF receptor is present in proteins involved in apoptosis and axonal guidance. *FEBS Lett* 371: 321-323.
140. Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* 24: 585-593.
141. Gallo G, Letourneau PC (2004) Regulation of growth cone actin filaments by guidance cues. *J Neurobiol* 58: 92-102.
142. Fundin BT, Silos-Santiago I, Ernfors P, Fagan AM, Aldskogius H, et al. (1997) Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. *Dev Biol* 190: 94-116.
143. Kinkelin I, Stucky CL, Koltzenburg M (1999) Postnatal loss of Merkel cells, but not of slowly adapting mechanoreceptors in mice lacking the neurotrophin receptor p75. *Eur J Neurosci* 11: 3963-3969.
144. Cronk KM, Wilkinson GA, Grimes R, Wheeler EF, Jhaveri S, et al. (2002) Diverse dependencies of developing Merkel innervation on the trkA and both full-length and truncated isoforms of trkC. *Development* 129: 3739-3750.
145. Fan L, Girnius S, Oakley B (2004) Support of trigeminal sensory neurons by nonneuronal p75 neurotrophin receptors. *Brain Res Dev Brain Res* 150: 23-39.
146. Yee C, Bartel DL, Finger TE (2005) Effects of glossopharyngeal nerve section on the expression of neurotrophins and their receptors in lingual taste buds of adult mice. *J Comp Neurol* 490: 371-390.
147. Krimm RF (2006) Mice lacking the p75 receptor fail to acquire a normal complement of taste buds and geniculate ganglion neurons by adulthood. *Anatomical Record Part A, Discoveries in Molecular, Cellular, & Evolutionary Biology* 288: 1294-1302.

148. Mistretta CM, Hill DL (2003) Development of the taste system: basic neurobiology. . In: R.L. D, editor. Handbook of Olfaction and Gustation. New York: Marcel Dekker. pp. 759-782.
149. Yeiser EC, Rutkoski NJ, Naito A, Inoue J, Carter BD (2004) Neurotrophin signaling through the p75 receptor is deficient in *traf6*^{-/-} mice. *J Neurosci* 24: 10521-10529.
150. Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, et al. (2000) A novel family of mammalian taste receptors. *Cell* 100: 693-702.
151. Nikolettou V, Lickert H, Frade JM, Rencurel C, Giallonardo P, et al. (2010) Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does not. *Nature* 467: 59-63.
152. Ben-Zvi A, Ben-Gigi L, Klein H, Behar O (2007) Modulation of semaphorin3A activity by p75 neurotrophin receptor influences peripheral axon patterning. *J Neurosci* 27: 13000-13011.
153. Huang T, Krimm RF (2010) Developmental expression of Bdnf, Ntf4/5, and TrkB in the mouse peripheral taste system. *Dev Dyn* 239: 2637-2646.
154. Zeng Q, Kwan A, Oakley B (2000) Gustatory innervation and bax-dependent caspase-2: participants in the life and death pathways of mouse taste receptor cells. *J Comp Neurol* 424: 640-650.
155. Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, et al. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *J Cell Biol* 143: 1691-1703.
156. Dechant G, Barde YA (1997) Signalling through the neurotrophin receptor p75NTR. *Curr Opin Neurobiol* 7: 413-418.
157. Barker PA, Lomen-Hoerth C, Gensch EM, Meakin SO, Glass DJ, et al. (1993) Tissue-specific alternative splicing generates two isoforms of the trkA receptor. *J Biol Chem* 268: 15150-15157.
158. Sandhya VK, Raju R, Verma R, Advani J, Sharma R, et al. (2013) A network map of BDNF/TRKB and BDNF/p75NTR signaling system. *J Cell Commun Signal*.
159. Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 72: 609-642.
160. Nimnual AS, Yatsula BA, Bar-Sagi D (1998) Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. *Science* 279: 560-563.
161. Qian X, Ginty DD (2001) SH2-B and APS are multimeric adapters that augment TrkA signaling. *Mol Cell Biol* 21: 1613-1620.

162. Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME (1998) Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol Cell Biol* 18: 1946-1955.
163. Ouwens DM, de Ruiter ND, van der Zon GC, Carter AP, Schouten J, et al. (2002) Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO J* 21: 3782-3793.
164. Shi GX, Han J, Andres DA (2005) Rin GTPase couples nerve growth factor signaling to p38 and b-Raf/ERK pathways to promote neuronal differentiation. *J Biol Chem* 280: 37599-37609.
165. Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, et al. (2005) Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. *Mol Cell Biol* 25: 336-345.
166. Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, et al. (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J Cell Biol* 146: 955-966.
167. Yamada M, Ohnishi H, Sano S, Nakatani A, Ikeuchi T, et al. (1997) Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. *J Biol Chem* 272: 30334-30339.
168. Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, et al. (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. *Mol Cell Neurosci* 14: 28-40.
169. Middleton G, Hamanoue M, Enokido Y, Wyatt S, Pennica D, et al. (2000) Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. *J Cell Biol* 148: 325-332.
170. Kong H, Boulter J, Weber JL, Lai C, Chao MV (2001) An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. *J Neurosci* 21: 176-185.

CURRICULUM VITAE

NAME: Da Fei

ADDRESS: Department of Anatomical Sciences and Neurobiology
500 S Preston St.
HSC Campus, University of Louisville
Louisville, KY 40202

EDUCATION: 2008-2013
Ph.D. Anatomical Sciences and Neurobiology
University of Louisville, Louisville, KY

1999-2004
B.A.
University of Science and Technology of China, Hefei, China

EXPERIENCE: 2004-2008
Lab Manager
Institute of Neuroscience, Shanghai, China

PUBLICATIONS: **Fei D** and Krimm RF. Genuiculate ganglion neurons remaining in the *TrkB*^{-/-} mice innervate and support the development of taste buds. (2013, in preparation)

Fei D and Krimm RF. The p75^{NTR} function as a positive factor in taste neuron survival and mediates taste nerve innervating the middle region of the tongue. (2013, in preparation)

Zhang XB, Jiang P, Gong N, Hu XL, **Fei D**, Xiong ZQ, Xu L, Xu TL. A-type GABA receptor as a central target of TRPM8 agonist menthol. (2008) *Plos One* 10: e3386

Zhang LH, Gong N, **Fei D**, Xu L, Xu TL. Glycine uptake regulates hippocampal network activity via glycine receptor-mediated tonic inhibition. (2008) *Neuropsychopharmacology* 3: 701-11.

MEETINGS:

Fei D and Krimm RF. p75^{NTR} regulates gustatory innervation patterning during development. (2013) *The 35th annual meeting of AchemS*, Huntington Beach, CA

Fei D and Krimm RF. The role of the neurotrophin receptors in gustatory development. (2011) *The 21st annual Neuroscience Day*, Louisville, KY

Fei D and Krimm RF. The role of TrkB receptor in peripheral taste development (2010) *The 32nd annual meeting of AchemS*, St. Pete Beach, FL