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The effect of neurotrophic factors on spiral ganglion neurons

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THE EFFECT OF NEUROTROPHIC FACTORS ON SPIRAL GANGLION NEURONS

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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ABSTRACT

The current study investigates the survival and neuritogenic effects of various neurotrophic factors on rat spiral ganglion neurons (SGNs) *in vitro*. In particular, ciliary neurotrophic factor (CNTF), glial derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and neurturin (NRTN) were assayed on postnatal day 4-6 SGNs. CNTF and NT-3 produced a robust survival effect while GDNF and NRTN failed to do so. A dose response revealed CNTF to be effective at promoting survival as low as 5ng/ml. In addition, CNTF promoted neurite growth in both depolarizing and non-depolarizing conditions, suggesting that CNTF can partially overcome the inhibitory effect of membrane depolarization. Lastly, the effect of NTFs was assayed between basal and apical neurons in culture. The preliminary results suggest there is no difference in response to NTFs between these two spatially distinct populations, however, it was noted that under depolarizing conditions apical neurons produce significantly shorter neurites than their basal counterparts

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CHAPTER 1

INTRODUCTION

<u>Anatomy and Physiology</u>

The auditory system is composed of central and peripheral assemblages that are connected together by highways of neuronal fibers. Like any biological system it is complex and a detailed description of all components and functions is both beyond the limit of this thesis and capability of the author. Rather, a simplified overview will be given with focus on the central players present in the current study. For the anatomical aficionado an excellent and detailed description can be found elsewhere (Slepecky, 1996). The peripheral auditory system is primarily housed in a concha shaped structure called the cochlea. Within this osseous casing are spiral ganglion neurons, the cells of which this study is primarily concerned. These bipolar neurons are the courier of sound information and as will be described they receive electrical input from specialized "hair cells" relaying their message to the central nuclei located in the brainstem. In general, the perception of sound is the result of successive energy transformations beginning at the tympanic membrane of the middle ear and ending in the temporal cortices of the brain. I will not mention the entire pathways involved; I will only mention two relevant steps. First is the deflection of stereocilia bundles located on the apical surface of hair cells. Deflection of these eponymous projections initiates an intracellular chain reaction in the hair cells beginning with inward potassium currents at the apical region and ending with the release of glutamate at the basal end. The second important event is the binding of glutamate by spiral ganglion neurons followed by membrane depolarization. If the neuronal membrane is sufficiently depolarized an action potential will manifest. This membrane depolarization, or "excitation" as it is commonly referred, occurs within a region of tissue called the organ of Corti. Embedded in the organ of Corti are three rows of outer hair cells (OHCs) and a single row of inner hair cells (IHCs). Each hair cell is

juxtaposed by supporting cells termed phalangeal and pillar cells which are thought to be important for the proper function and maintenance of both surrounding hair cells and SGNs. Upon sufficient excitation the action potential is propagated afferently passing through the neuronal cell body and continuing down its central axon where it bifurcates and synapses on the cochlear nuclei of the brainstem. SGNs are classified into two groups depending on the type of hair cell they contact. The majority, type I, synapse on the inner row of hair cells (IHC) while the remaining type II synapse on the outer row hair cells (OHC). The cell bodies of SGNs reside within a region of tissue anatomically identified as Rosenthal's canal. This canal houses a cluster of neuronal and non-neuronal cell bodies collectively called the spiral ganglia. The unique structure of the cochlea is the result of the ganglion spiraling upward from the base and tapering near the apex.

In addition to receiving glutamatergic input from hair cells SGNs receive neurotrophin support from the same mechanosensory cells. As a result, the integrity of HCs is viewed as critical for the function and maintenance of spiral ganglion neurons. Despite this dual role, hearing can still be restored after HC damage by surgical insertion of hearing prosthetics such as cochlear implants. In contrast, the death of SGNs is indispensable and results in irreversible hearing loss. Thus, understanding the factors that maintain the survival of neurons is critical for the preservation of hearing. Indeed, the present study is an investigation of potential survival factors for SGNs. Specifically, the trophic capabilities of GDNF, NRTN and CNTF were assayed and analyzed on spiral ganglion neurons *in vitro*.

<div align="center">Neurotrophic Factors</div>

Hints of the first neuronal trophic factor was revealed following the observation that grafts of sarcoma tissue implanted into chick limbs promoted the survival and growth of chick sympathetic neurons (Levi-Montalcini & Hamburger, 1951; Cohen et al., 1954). This unknown factor was subsequently purified from snake venom and christened Nerve

Growth Promoting Factor (NGF) (Cohen & Levi-Montalcini, 1956). Since then a variety of neurotrophic factors have been discovered with effects ranging from differentiation, survival and maintenance of PNS and CNS neurons. These soluble proteins are synthesized and secreted by neuronal and non-neuronal cells. NGF is now classified within a larger family of structurally related proteins called neurotrophins. The following study focuses on a variety of proteins representative from three distinct families of neurotrophic factors. The trophic factors consist of the following: ciliary neurotrophic factor (CNTF), Glial-Derived neurotrophic factor (GDNF), neurturin (NRTN), neurotrophin-3 (NT-3) and Brain derived neurotrophic factor (BDNF). A brief review of the ligands, receptors and signaling pathways is given below.

CNTF is part of the cytokine family, a family traditionally involved with the affairs of inflammation and immunity. CNTF signals through a tripartite receptor complex comprised of leukemia inhibitory factor receptor (LIFR), glycoprotein 130 (gp130), and CNTFRα. CNTF binds specifically to CNTFRα resulting in the recruitment of LIFR and gp130. CNTFRα does not contain a transmembrane domain and is instead tethered to the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety. As a result, intracellular signaling cannot occur directly through CNTFRα, rather, the ternary complex signals through the cytoplasmic domains of LIFR and gp130 resulting in the activation of the JAK/STAT signaling pathway (Heinrich et al., 2003).

NT-3 and BDNF are part of the neurotrophin family. Unlike CNTF, these proteins signal as homodimers through a transmembrane tyrosine kinase receptor called tropomyosin-receptor kinase (Trk). The receptor for BDNF and NT-3 is TrKB and TrkC, respectively. Upon binding of the ligands, Trks dimerize and cross-phosphorylate each other. The intracellular domains of Trk contain adaptor binding sites resulting in the activation of multiple proteins and signaling pathways. Examples of activated proteins include Akt/PKB, ERK, PLCγ, PKC and others. Activation of these pathways typically leads to trophic effects in the cell. Furthermore, neurotrophins can signal through p75[NTR],

a low affinity neurotrophin receptor that can antagonize the effects of Trk signaling (Reichardt, 2006).

GDNF and NRTN are part of the GDNF-family of ligands (GFLs). Both of these GFLs signal as homodimers and bind to their receptors GFRα1 and GFRα2, respectively. Because all GFL receptors lack an intracellular domain these GPI-linked receptors require the presence of the transmembrane protein RET. Like Trk, RET is a receptor tyrosine kinase and can self dimerize resulting in its phosphorylation and subsequent binding of adapter proteins such as Shc (Src-homologous and collagen-like protein), FRS2 (fibroblast growth factor receptor substrate 2), GRB2 (growth factor receptor-bound protein 2) and others. Indeed, RET shares many of the signaling pathways characteristic of Trks (Airaksinen & Saarma, 2002). In addition to RET, GFRαs can signal through neural cell adhesion molecule (NCAM) resulting in the activation of intracellular proteins such as Fyn and FAK (Paratcha et al., 2003).

<p style="text-align:center"><u>HC Derived NTF-dependence Hypothesis</u></p>

The prevailing view regarding the integrity of mature SGNs asserts that neurons require HC-derived NTF for their survival. This view neatly explains the death of neurons following trauma to the organ of Corti. The view is as follows, when hair cells are damaged, such as occurs during exposure to loud noise or ototoxic agents, SGNs are deprived of their hair cell derived neurotrophins. The result is neurite retraction and apoptotic death. This view has been supported by three general observations. First, the development and differentiation of SGNs is dependent upon the secretion of neurotrophins produced by sensory cells the organ of Corti. Secondly, exogenous application of neurotrophins in the adult cochlea can ameliorate the death of neurons after induced hearing loss and lastly, histological biopsies from both animal and human temporal bones have observed a correlation between the loss of SGNs and lesions of HCs. However, as will be subsequently addressed, recent data has emerged conflicting

with this long established view but first an overview of the dogma regarding the dependence of hair cell derived neurotrophins will be given.

SGN Development and Neurotrophins

The use of *in situ* and lacZ "knock ins" has revealed NT-3 and BDNF to be expressed in the organ of Corti as early as embryonic day 11. Expression remains present at birth and is extended into adulthood albeit lower levels (Pirvola et al., 1992; Ylikoski et al., 1993; Schecterson and Bothwell, 1994; Wheeler et al., 1994; Sugawara et al., 2007). The expression patterns of NT-3 and BDNF along the tonotopic axis are not identical. During development NT-3 is believed to be highly expressed in both hair cells and supporting cells in a basal to apical gradient, whereas BDNF expression is believed to be primarily enriched in hair cells in an apical to basal gradient (Farinas et al., 2001). In adult mice the expression intensity of NT-3 decreases and BDNF expression decreases to almost undetectable levels (Wang and Green unpublished; Bailey and Green unpublished). Furthermore, the direction of the NT-3 gradient is reversed in the adult cochlea. That is, NT-3 is expressed in an apical to basal gradient (Schimmang et al., 2003; Sugawara et al., 2007). Not everyone agrees with the exact expression pattern of neurotrophins as some studies employing *in situ* techniques have failed to observe this dynamic pattern (Ylikoski et al., 1993; Wheeler et al., 1994). However, the apical to basal gradient of NT-3 can partially explain the phenomenon that neuronal loss occurs to a greater extant in the base than the apex following sensory damage. In this model apical SGNs are exposed to more NT-3 than their basal counterparts thus increasing their ability to survive in the face of trauma.

In addition to the expression of BDNF and NT-3, *in situ* hybridization has also revealed the presence of TrkB & TrkC in the developing spiral ganglia in *vivo* (Ylikoski et al., 1993; Pirvola et al.,1994) as well as *in vitro* (Mou et al., 1997). The Expression of the low affinity neurotrophin receptor $p75^{NTR}$ has also been observed in the spiral ganglia

(Ylikoski et al., 1993; Schecterson and Bothwell, 1994). In the adult, however, p75^{NTR} does not appear to be localized to neurons but rather in the spiral ganglion Schwann cells (Provenzano et al., 2011). The role of p75^{NTR} in development and in the mature cochlea is not completely understood.

The observation that NT-3 and BDNF are expressed in the sensory epithelia while their receptors are expressed on SGNs implies that developing neurons rely on neurotrophins for proper innervation and survival. Genetic ablation of HCs during development disrupts SGN innervation demonstrating the importance of HCs on SGNs (Pan et al., 2012). Further support of the view that SGNs require neurotrophins for survival has come from *in vitro* experiments. Exogenous application of NT-3 and BDNF can promote the survival of cultured neonatal neurons (Lefebvre et al., 1994; Malgrange et al., 1996; Hegarty et al., 1997; Mou et al.,1997). However, direct *in vivo* evidence for the requirement of neurotrophins can only come from transgenic mice lacking NT-3, BDNF, TrkB and/or TrkC. Indeed these mice exist and lack of either receptor or ligand leads to a reduction in the number SGNs. However, the results and interpretations of single KO experiments are complicated and varied among research groups. Undeniable evidence regarding the requirement of neurotrophins during development has come from transgenic mice harboring double KOs of either the NT-3/BDNF and or TrkB/TrkC loci (Ernfors et al., 1995). In these mice SGN degeneration occurs quickly over a short period of time resulting in consummate death of all SGNs. For a comprehensive review regarding the development of SGNs and expression of neurotrophins see Fritzsch et al, (2004).

<div align="center">Infusion of Neurotrophins Rescues SGN Death</div>

The observation that developing SGNs rely on neurotrophins released by HCs has suggested that adult SGNs also rely on neurotrophins for survival. Unfortunately, a strict interpretation of the double KO experiments is limited to the context of cochlear

development. Indirect evidence for the requirement of neurotrophins in adult SGNs has

come from observations that infusion of neurotrophins into the adult cochlea can

ameliorate the death of SGNs after deafening. Many of these experiments utilized guinea

pigs as a model system and osmotic pumps as a mode of delivery. Infusion of either NT-3

or BDNF into the scala tympani results in the amelioration of neuronal death after

treatment with aminoglycosides (Ernfors et al., 1996; Staecker et al., 1996; Miller et al.,

1997; Wise et al., 2005). However, it is important to keep in mind that response to

exogenous neurotrophins is not direct evidence for their necessity.

<center>Organ of Corti Lesions and Neuronal Loss</center>

Perhaps the oldest evidence suggesting the requirement of HC-derived NT-3 for

mature SGNs comes from histological preparations of humans and animal temporal

bones. In the 1950's patients with tuberculosis and benign ear infections were treated

with neomycin and/or kanamycin. These broad acting antibiotics soon revealed their true

range of potency. In addition to eradicating the infection these antibiotics caused

profound hearing loss in patients (Carr et al., 1950, 1951; Frost et al., 1959). Follow up

studies on aminoglycoside-treated animals began piecing together a general sequence of

pathology. Administration of antibiotics initially produced lesions in hair cells and/or

atrophy of the organ of Corti. This havoc was quickly followed by the retraction of

neurites and eventual degeneration of neurons (Schuknecht et al., 1965; McFadden et al.,

2004). An old review by Kohonen (1965) neatly summarizes the destructive power of

ototoxic drugs:

> "The pattern of neural damage was found to follow very closely
> the damage to the sensory cells. Degeneration of nerve endings or
> nerve fibers appears very soon after the loss of corresponding hair
> cells, and neural damage was never found without loss of sensory
> cells".

Thus, began the idea that the survival of SGNs largely depends on the presence of HCs,

or at the very least the integrity of the organ of Corti.

HC Derived NTF-Independence Hypothesis

Importance of Supporting Cells

The advent of molecular biology and genetically engineered mice has allowed investigators to gain unprecedented insights into the molecular underpinnings of development, physiology and pathology. Consequently our understanding of auditory neurobiology is constantly being update and at times refurnished. To understand the role of neuregulin signaling within the cochlea Stankovic and colleagues used transgenic mice expressing a dominant-negative erbB4 receptor under the GFAP promoter (Stankovic et al., 2004). This allowed the investigators to completely abolish neuregulin-ErbB signaling in supporting cells of the organ of Corti. The result was spectacular to say the least; 80% of neurons died. Equally interesting was the observation that neuronal death began after development at approximately three weeks post birth. Moreover, there was a significant reduction of NT-3 levels despite the presence of intact hair cells. This suggests that neuregulin signaling in supporting cells contributes to the expression of NT-3. Indeed a relationship between neuregulin signaling and NT-3 expression has been observed in other systems (Verdi et al., 1996). The study by Stankovic, however, was not able to determine whether the decrease of NT-3 was the result of decreased release of NT-3 by hair cells or decreased release of NT-3 by supporting cells. In either case, the study clearly demonstrates the necessity of ErbB signaling in supporting cells for the integrity of SGNs.

Further evidence for the importance of supporting cells has come from careful analysis of chinchilla and cat temporal bones after aminoglycoside and carboplatinum treatment. These temporal bones revealed that neuronal survival was enhanced under the presence of inner pillar and inner phalangeal cells (Sugawara et al., 2005). Similar conclusions regarding the importance of supporting cells have also been drawn from human temporal bones (Johnsson, 1974; Suzuka & Schuknecht, 1988; Linthicum &

Fayad, 2009). Together these findings lend a greater rôle to the often overlooked *supporting* cells of the organ of Corti.

A critic to the use of noise or pharmacological agents for destroying hair cells is that the lesions typically extended beyond hair cells destroying nearby supporting cells (Ding et al., 1999). As a result it is difficult to disentangle the effects of hair cells loss from that of supporting cell loss. To bypass the collateral damage caused by aminoglycosides Zilberstein and colleagues developed a transgenic mouse line that allowed them to selectively destroy hair cells at any given age merely by altering the amount of thiamine in the diet. (Liberman et al., 2006; Zilberstein et al., 2012). Amazingly, but perhaps not too surprisingly, the loss of hair cells had no effect on the integrity of neurons. In this model, supporting cells were intact and appeared functional. Unfortunately this study did not assay the levels of NT-3 following ablation of hair cells. It would have been interesting to observe how much NT-3 levels changed after the selective loss of hair cells.

Delayed Neuronal Death

Unlike the quick death of developing SGNs observed in the double KO of NT-3/BDNF, death of SGNs in the mature cochlea is much more gradual and delayed. Treatment with aminoglycosides or intense noise exposure can result in hair cell destruction and an associated decrease of NT-3 expression. This havoc is followed by damage to synapses, retraction of neurites and eventual death of SGNs. (Spoendlin, 1975; Pujol et al., 1985; Kujawa & Liberman, 2006). The time course of SGN death varies depending upon the organism and method of deafness. For example, in cats the time course of degeneration following HC loss can span years (Ylikoski et al., 1974; Kiang et al., 1976; Leake & Hradek, 1988) and up to months in rats (McFadden et al., 2004; Alam et al., 2007). In humans neurons can survive for decades after hair cell loss (Linthicum & Fayad, 2009). If HC-derived neurotrophins are the sole source of neurotrophic factors for

SGNs then why is death delayed after HC death? One explanation is that neurons may be receiving trophic support from other regions. In agreement with this idea is the observation that phoso-CREB is present in approximately 30% of SGNs long after deafening, suggesting that the remaining neurons are still receiving neurotrophic support (Alam et al., 2007). Results such as these have spurred interest in the quest the uncover the hidden sources of NTFs.

Expression of Neurotrophins Outside Organ of Corti

Trophic support need not be restricted to regions such as the organ of Corti. It is possible that other target sites may serve as an alternative source of trophic signaling. Indeed mRNA expression of NT-3 and BDNF has been observed in the cochlear nucleus (Lefebvre et al., 1994) Furthermore, a paper by Mariach and colleagues demonstrated that disruption of the cochlear nucleus and accessory auditory nuclei results in a loss of SGNs beginning at P3 suggesting that the cochlear nucleus may serve a trophic role for neonatal neurons (Maricich et al., 2009). Finally, the spiral ganglion itself may serve as a sink of neurotrophins. In culture, at least, it has been observed that Schwann cells are capable of producing BDNF and NT-3 (Hansen et al., 2001a, 2001b) and microarrays have demonstrated the presence of these NTFs in the spiral ganglia (Bailey et al., 2012).

Expression of Non-neurotrophins

The availability of neurotrophic factors is not limited to the neurotrophin family. Non-neurotrophins have been observed in the organ of Corti, spiral ganglia and cochlear nucleus. Recent QT-PCR and microarray data (Bailey et al., 2012) has demonstrated the presence of CNTF and NRTN in the aforementioned regions of the mature cochlea. These same microarrays have also revealed the presence of their cognate receptors in the spiral ganglia and are in agreement with older studies (Ylikoski et al., 1998; Stankovic and Corfas 2003). In addition, *in situ* of CNTF and GDNF mRNA has also been reported

in the cochlear nucleus and inner hair cells of rats, respectively (Malgrange et al., 1998; Hafidi et al., 2004; Ylikoski et al., 1998).

<div align="center">Effect of Non-neurotrophins on SGN Survival</div>

The expression of GDNF, NRNT and CNTF mRNA in the cochlea suggest that these factors are trophic to SGNs. Indeed *in vitro* assays have demonstrated survival effects for both CNTF and GDNF (Hartnick, et al., 1996; Ylikoski, et al., 1998). Furthermore, cochlear infusion of GDNF has been shown to ameliorate SGN death following deafning (Ylikoski, et al., 1998; Yagi, et al., 2000; Kanzaki, et al., 2002). Unlike GDNF, NRTN has not been observed to exert a trophic effect on chick SGNs (Hashino et al., 1999). However, with respect to the effect of CNTF and GDNF there is some inconsistancy in the literature regarding their survival promoting abilities. For example, CNTF failed to promote survival of organotypic cultures whereas it promoted the survival of dissociated neurons (Staecker, et al., 1995; Hartnick, et al., 1996). In regard to GDNF, this study was not able to repoduce the survival effects previously observed.

<div align="center">Neurite Growth and Cochlear Implants</div>

Maintaining the survival of residual SGNs is a major focus for cochlear implant technology, indeed the previous pages have completely centered on the survival aspect. However, another focus of CI research is the mechanisms and factors that promote SGN neurite growth. Regenerating neurites towards cochlear electrodes is a primary objective because it is believed that re-innervation of SGNs into the organ of Corti could potentiate the efficacy of CIs. However, the cost of stimulating SGNs bears a high price. The pro-survival effect of membrane depolarization also inhibits neurite growth (Hansen et al., 2003). Thus, understanding how depolarization inhibits neurite growth is essential if one hopes to bypass this undesired side effect. The pro-survival effects of depolarization have been shown to be partly mediated by voltage gated Ca^{2+} channels, cAMP, PKA, CaMKII

and CaMKIV (Hegarty et al., 1997; Hansen et al., 2001, 2003; Xu et al., 2012). Thus, attempts to understand the process by which electrical excitation regulates neurite growth have initially focused on the role of these proteins. Of particular interest are the Ca^{2+}/Calmodulin-dependent Kinases (CaMKs). These multifunctional holoenzymes are activated by intracellular Ca^{2+} and the spectrums of their functions are vast ranging from LTP, synaptogenesis and neurite elongation (Malenka et al., 1989; Malinow et al.,1989; Zou & Cline, 1996). For example, in hippocampal neurons CaMKIIβ but not the CaMKIIα isoform regulates neurite extension and synapse formation (Fink et al., 2003). To understand the relationship between depolarization and neuritegenesis in SGNs, Hansen and colleagues (2003) investigated the role of CaMKII and CaMKIV. Unlike hippocampal neurons, SGNs transfected with constitutively active CaMKIIα result in a reduction of neurite lengths, suggesting that physiological CaMKIIα normally inhibits neurite growth. To confound matters, inhibition of CaMKII by use of a peptide inhibitor failed to rescue neurite inhibition under depolarizing conditions (Roehm et al., 2008). Thus, it is not clear how CaMKII is contributing to neurite inhibition. One explanation is that different levels of CaMKII regulate different aspects of neurite growth. Indeed, this biphasic influence of CaMKII is observed on spine formation in hippocampal slices (Zha et al., 2009). For example, high levels of CaMKII activity, induced by epileptiform activity, do not contribute to spine loss on dendrites; whereas under basal conditions normal activity of CaMKII does contribute to spine loss. Another and perhaps less ambiguous mediator of neurite regulation is the calcium activated protease, calpain. It was demonstrated that depolarization of the membrane activates calpain and its inhibition was seen to partially rescue neurite inhibition (Roehm et al., 2008).

The roles of cAMP and PKA have also been frustrated (Xu et al., 2012). Overexpression of constitutively active GFP-PKA in SGNs results in a reduction of neurite lengths whereas inhibition of PKA under depolarizing conditions does not rescue the neurite phenotype. Investigation of cAMP was found to exert a biphasic effect on

neurites; at low and high concentrations it inhibits growth but promotes neurite growth at intermediate levels. In summary, our understanding of neurite growth in SGNs is infantile but growing. More work is needed to understand the complexity of neurite regulation if one ever wishes to regenerate neurites towards CI electrodes.

The current study investigates the neurite promoting effects of NTFs. CNTF was found to be a strong promoter of neurite growth under depolarizing and non-depolarizing conditions. Therefore, CNTF may have application to CI research. The ability of CNTF to bypass the undesired neurite effects of depolarization is exciting, unfortunately, little is known about how this may occur and this study does not address any mechanism. As a cytokine CNTF can activate JAK/STAT signaling and so neurite growth may be directly dependent upon transcription. In addition, CNTF can regulate MAPK/ERK signaling, pathways well-known to regulate axonal growth and cytoskeletal rearrangements in cells (Vaudry et al., 2002).

<div align="center">Experimental Design</div>

The microarray and QT-PCR performed by Bailey (2012) demonstrate that GDNF, NRTN, and CNTF expression remain present after deafening. This finding suggests that these proteins may serve as trophic factors to the remaining neurons after destruction of hair cells. This could explain why some neurons persist after deafening. The current study does not directly test this hypothesis; rather, it addresses the question whether SGNs can respond to GDNF, CNTF and NRTN *in vitro*. As previously mentioned experiments of this nature have been carried out yet the results of these experiments have been either negative or conflicting. To the best of my knowledge this study attempts for the first time to assay the neurite and survival effects between cultured SGNs isolated from apical and basal regions. In addition, this study assays the neurite effects of CNTF under depolarizing conditions, a condition that has been shown to inhibit neurite growth.

It should be mentioned that similar culturing techniques involving the separation of apical and basal neurons have been performed. These studies, however, did not look at survival and neurite growth but rather at differences in electrophysiological phenotypes (Adamson et al., 2002). The results of this thesis demonstrate that NRTN and GDNF do not promote survival whereas CNTF produces a robust response. It is also shown that CNTF promotes neurite growth under depolarization and non-depolarization conditions. As for the "apical vs. basal" experiments both populations of neurons appeared to respond equally well to NTFs. However, it was observed that apical neurons produce significantly shorter neurites than their basal counterparts. Indeed this was an unexpected finding and to my knowledge has never been reported.

Because it is not currently possible to culture dissociated neurons from older rats dissections were performed on postnatal day 5. This age was chosen because the bony structure of the cochlea is still soft and malleable thus allowing easy access to neurons. It is worth mentioning that this period is before the onset of hearing (Wada, 1923; Jewett & Romano, 1972). Despite the inabllity to hear, cellular connections between neurons and hair cells are already established (Pujol et al., 1998). Thus, given the technical restraints, postnatal day 5 is an optimal day to perform dissection. The use of a dissociated cell culture system is well suited to test the trophic ability of proteins. Parameters such as protein concentration can be regulated. In addition, subsequent steps such as imaging, counting cells and measuring neurite lengths are relatively easy in a dissociated cell system. An example of a dissociated culture after three days in membrane depolarizing media is depicted below in figure 1. The image on the left is representative of a culture viewed under bright field microscopy. An HRP conjugated secondary antibody was used to label NF200 in neurons. The image on the right utilized a fluorophore conjugated secondary and was the preferred technique employed to score neurons and measure neurite lengths.
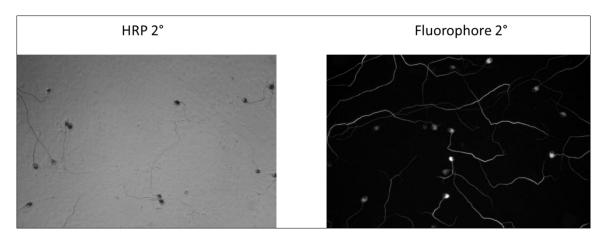
Figure 1. Representative images of SGNs after 3 days in culture. Bright field microscopy (left). Fluorescence microscopy (right). SGNs are labeled against NF200.

CHAPTER 2

METHODS

Tissue culture plates: Neurons were cultured in 96-well tissue culture dishes (Falcon). Plates were pre-coated with poly-ornithine (0.1 mg/ml in 10 mm borate buffer, pH 8.4) for 1hr at room temperature. Wells were then washed with distilled H_2O three times and allowed to air dry. Plates were then coated with laminin (mouse EHS, Boehringer Mannheim and Life Technologies, 20 μg/ml in HBSS) overnight at 4°C.

Culture media: Plating media and 25K media consisted of 8.5 ml of high-glucose (4.5 mg/ml) DMEM, 100μl of penicillin (0.1 mg/ml), 100μl of streptomycin (0.1 mg/ml), 100μl of N2 formulation which consist of human apo-transferrin (100 μg/ml), putrescine (100 μm), progesterone (20 nm), selenium (30 nm), crystalline BSA (20 μg/ml), and d-glucose (1.5 mg/ml), 50μl of fresh insulin (10 μg/ml) and 1.5 ml of 160mM potassium.

Cochlear Dissection: Postnatal day 4-6 Sprague Harley rat pups were used for experiments that did not consist of separating basal and apical neurons. The "base vs. apical" experiments used only P5 rats. All protocols were approved by the University of Iowa Animal Care and Use Committee. The protocol was followed similarly to the protocol described by Hegarty et al., 2007. The current protocol is as follows: rats were aseptically wiped with 70% alcohol and placed in ice for 3-5 minutes. Pups were then quickly decapitated with sterile scissors and a longitudinal cut was made separating the two cerebral hemispheres. The temporal bone was removed and placed in ice-cold PBS. Removal of extraneous tissue from the temporal bone was performed in ice-cold PBS under a dissection scope at 2X magnification. After removal of tissue the external osseous matter was carefully peeled away. Next the stria vascularis was removed followed by the organ of Coti. After successful removal of unwanted tissue the spiral ganglia and modiolus were collected in ice cold Ca^{2+}/Mg^{2+}-free HBSS. For the "apex vs.

base" experiments the spiral ganglia was divided into thirds. The middle third was discarded while the top and bottom thirds were kept for experimentation.

Enzymatic and mechanical dissociation: The spiral ganglion was spun down quickly and the HBSS was removed. .1% Trypsin and .1% collagenase in Ca^{2+}/Mg^{2+}-free HBSS were added to the ganglia. The ganglia were incubated for 30 min in a 37°C water bath. After incubation 10% FBS was added to terminate the enzymatic reaction. The mixture was placed on ice for 5 min followed by careful removal of the supernatant. The remaining mixture was diluted 1/100 fold by addition of 25K so as to dilute the protease mixture. The dilution was followed my mechanical dissociation using a p1000 pipetteman. The ganglia were triturated approximately10-20 times. The debris was allowed to settle and the supernatant was carefully removed and placed in a sterile culture tube. NT-3 was to the supernatant for a final concentration of 50ng/ml. 100ul of the suspension was added to wells. For the "Apex vs. Base" experiment the concentration of basal and apical cell suspension was determined by use of a hemacytometer. To determine viability of cells.1% Trypan Blue was added to the aliquot used for the hemacytometer. Concentration was determined and the suspensions were diluted appropriately so as to yield equal plating density. Cells were grown in 140 μl medium per well at 37°C in a 6.5% CO2 incubator

Staining: Cultures were fixed in fresh 4% PFA for 20 min. Washed three times in PBS. All subsequent steps were interspersed with PBS washing. Permeabilization was done using a non-ionic detergent (.2%Triton X-100 for 30 min). A goat serum based blocking buffer was added to wells at room temperature for 2 hours. A cocktail of primary antibodies mouse NF200 (1:1000), mouse 2H3 (1:1000) and mouse TUJ1 (1:1000) were added overnight in 4°C at a dilution of 1:1000. Secondary antibody consisted of fluorophore-conjugated anti mouse (1:1000) overnight at 4°C.

Imaging & Data Analysis: Images of wells were captured using an inverted Zeiss microscope using Open Lab software. Adobe Photoshop was used to create composite

images of wells. Image J software was used to count SGNs per well and measure neurite lengths.

CHAPTER 3

RESULTS

Prosurvival Effect of NTFs on SGNs (Entire Cochlea)

The survival effect of GDNF, CNTF, NRTN and NT-3 was assayed on SGNs representing regions of the entire cochlea. SGNs were dissected out from postnatal day 4-6 rat pups as described under methods. Briefly, dissociated neurons were plated in a 96-well tissue culture dish in membrane-depolarizing media (25K) containing 50ng/ml of NT-3. 24 hours post-plating cells were washed three times with 5K media resulting in a 1/64 dilution of initial plating conditions. NTFs were subsequently added to wells at a final concentration of 50ng/ml. 72 hours after addition of NTFs cells were fixed and stained for NF200. The number of neurons/well was determined and the survival was indexed as a percentage of the neurons/well in the 25K condition. Each biological experiment contained 2-3 technical replicates.

Both NT-3 and 25K have been previously reported to promote survival of SGNs *in vitro* (Hegarty et al., 1997) thus NT-3 served as an internal positive control throughout my experiments. As expected NT-3 significantly promoted survival at approximately 50% of 25K. The survival value of NT-3 is agreement with prior studies (Hansen et al., 2001).

The results demonstrate that CNTF promotes survival of SGNs *in vitro* while GDNF and NRTN do not. The survival of neurons exposed to CNTF was approximately 65% of that in the positive control. The finding presented in figure 2 is contrast with a previous study led by Staecker and colleagues (1995). In their study, CNTF (25ng/ml) was not able to promote survival of SGNs in organotypic cultures (Staecker et al., 1995). Though NT-3 appears less efficacious than CNTF, there was no significant difference between these two conditions.
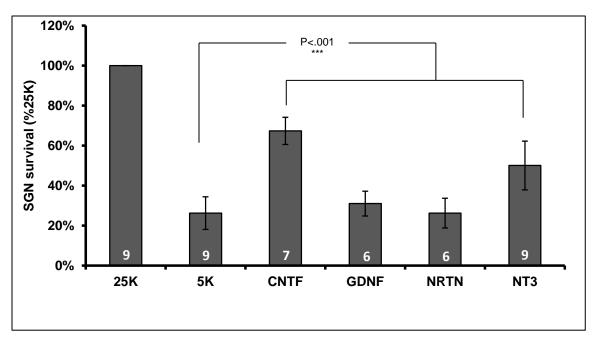
Figure 2. Survival effect of NTFs on SGNs *in vitro*. SGNs were treated with NTFs at a concentration of 50ng/ml. Survival is represented as a percentage of cells relative to 25K. Numbers in white represent the number of biological repeats. Error bars represent standard deviation. [ANOVA results: P<.001 for CNTF vs. 5K, NT-3 vs. 5K]

<u>Dose Response of Neurotrophic Factors</u>

The lack of survival upon addition of GDNF and NRTN (see figure 2) begs the questions whether an appropriate concentration was being utilized. On occasion biological response to ligands has been observed to follow bell shaped curves. For example, NGF can promote neuritogenesis in peripheral neurons after axotomy while high concentrations of NGF inhibit growth (Kemp et al., 2011). In general the bell shaped response may be a consequence of activating lower affinity receptors or different intracellular pathways. Presumably other NTFs may produce a similar bell shaped curve across a given concentration range.

To test whether SGNs can respond to GDNF and NRTN at larger or smaller concentrations a dose response over a 30 fold range was carried out for NTRN, GDNF and CNTF (Figure 3). The survival assay was performed similarly to that employed for

figure 2. The results demonstrate that CNTF can exert a survival effect as low as 5ng/ml

but not below .5ng/ml. The results of the dose response are in contrast with the finding by

Hartnick (1996). In the study by Hartnick CNTF was only capable of promoting survival

at a concentration of .01ng/ml. Figure 3 demonstrates that SGN's do not respond to

CNTF at or below .5ng/ml. Furthermore, a dose response of CNTF does not produce a

bell shaped response on SGNs. Figure 3 demonstrates that SGNs are unresponsive to

NRTN and GDNF across this 30 fold concentration range. Unlike figure 2, survival is

index as the ratio of the average neurons/well in the experimental condition to that of the

control condition (5K alone).
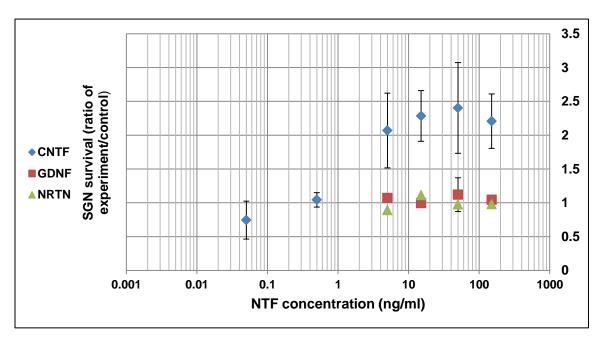

Figure 3. NTF dose response. SGN survival assay was performed over a 30 fold

concentration range. SGNs responded to CNTF as low as 5ng/ml. There is no response of

SGNs to GDNF and NRTN across the given range. Survival is index as the ratio of the

average neurons/well in the experimental condition to that of the control condition (5K

alone). Error bars represent standard deviation.

The 5ng/ml to 150ng/ml concentration range was chosen because many growth factors are thought to be active in the 1ng/ml – 10ng/ml range (Alberts et al., 2002; Freshney, 2005). This is only an estimate and the true concentrations of NTFs *in vivo* can exceed beyond these values. It is feasible that my range is too narrow and that SGNs respond to GDNF and NRTN at lower concentration values.

<center>Superior Cervical Ganglion Survival Assay</center>

The superior cervical ganglion is a cluster of sympathetic neurons that innervate the head, neck and heart. It has long been established that NGF, GDNF and NRTN can promote the survival of this population of neurons both in culture and *in vivo* (Thoenen et al., 1971; Banerjee et al., 1973; Buj-Bello et al., 1995; Kotzbauer, et al., 1996; Moore et al., 1996; Granholm et al., 1997). To rule out the possibility that the lack of effect of GDNF and NRTN is due to a faulty batch of protein a survival assay was carried out on these neurons using the same batch of GDNF and NRTN. Figure 4 demonstrates the results of a single biological experiment. Neurons were dissected and isolated following a standard protocol (Zareen & Greene, 2009). Briefly, cells were plated in a 96-well tissue culture dish. 24 hours after plating the media was washed and exchanged with NTF-containing media. During this exchange period a subset of wells were fixed. This was done so as to determine the average number of neurons/well (plating condition). The remaining wells were cultured with NTFs for an additional 72 hours. Survival is indexed as the number of neurons per well. Though statistical power is lacking, the preliminary results strongly show a survival effect of GDNF at 50ng/ml and 150ng/ml. NRTN also produced a survival effect as low as 15ng/ml. All these conditions show a greater number of neurons per well over the negative control condition (-NGF). NGF served as an internal positive control and wells treated with 100ng/ml of NGF maintained survival with greatest efficacy. Thus, it can be concluded that my batch of GDNF and NRTN retain biological activity and the possibility that these proteins are degraded is unlikely.

As discussed in the introduction, the preferred receptors for GDNF and NRTN are GFRα1 and GFRα2, respectively. In addition to NCAM signaling, there is also evidence that GFRα1 can uniquely signal via Src (Poteryaev et al., 1999; Trupp et al., 1999). To add to this complexity, experiments utilizing ^{125}I-labeled ligands demonstrated that GDNF can also bind to GFRα2 and NRTN to GFRα1 (Scott & Ibanez, 2001). Taken together, high concentrations of GFLs can signal through RET, NCAM, and Src. This promiscuity in GFRα-signaling does not undermine my conclusion regarding the integrity of my proteins but caution should be taken when interpreting dose response curves using high GFL concentrations.
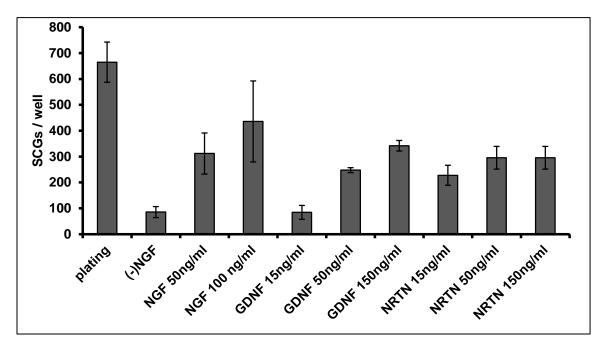

Figure 4. NTF survival assay on P1 rat SCG. Survival is represented as the average number of neurons per well. NGF served as a positive control. GDNF and NRTN were applied at 15, 50 and 150ng/ml. GDNF promoted survival at 50ng/ml and 150ng/ml while NRTN promoted survival over entire concentration range. Error bars represent standard deviation [N=1, 3 technical repeats.]

Effect of NTFs on SGN Neurite Growth (Entire Cochlea)

In addition to promoting survival, neurotrophic factors can also promote neurite growth both in culture and *in vivo* (Markus et al., 2002; Wise et al., 2005). As CNTF and NT-3 were the only neurotrophic factors to promote survival (figure 2) neurite measurements were focused on conditions involving these two trophic factors. Due to time constraints not every single condition used for figure 2 had their neurites measured. The bar graph of Figure 5 represents the results of neurite lengths after three days in culture. A representative image of SGNs in 25K and CNTF is given in figure 6. To summarize, 25K produced the shortest neurites while 5K + CNTF produced the longest neurites. Furthermore, 25K + CNTF produced significantly longer neurites than 25K alone. Together the results suggest that the inhibitory growth effects caused by depolarization can be partially overcome by CNTF. In addition, CNTF was observed to be more effective than NT-3 at promoting neurite growth, despite the similar ability to promote survival.

The neurite promoting effects of CNTF have been previously reported for SGNs *in vitro* (Hartnick, et al., 1996). In Hartnick et al., neurite lengths were quantified as the ratio of neurite lengths to the diameter of the organotypic explant. Therfore, it is not possible to directly compare my neurite measurements to their ratio values. Despite the difference in methodology CNTF promoted neuritogenesis in both cases. The observation that 5K produces significantly longer neurites than 25K is not a new finding (Roehm et al., 2008).

One explanation is that 25K actively inhibits neurite growth. As a result neurons in 5K are relieved from this inhibitory pressure. It should be reminded that neurons remaining in 5K are few (Figure 2), yet these neurons have somehow managed to stay alive in trophic-lacking conditions while neurons present in trophic-depolarizing conditions are abundant (figure 2). One explanation as to why few SGNs remain present
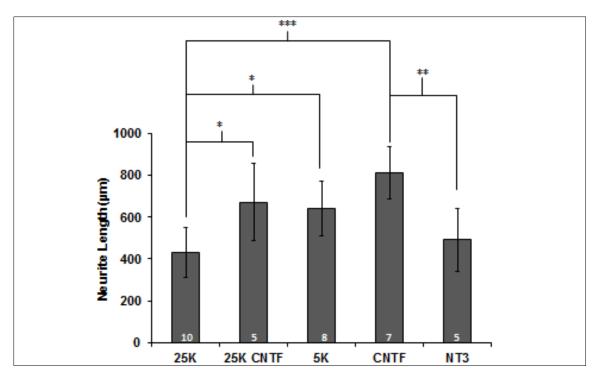
Figure 5. The effect of CNTF on neurite growth. Neurite lengths were measured after three days exposure to CNTF (50ng/ml) and NT-3 (50ng/ml). White number denotes number of biological repeats. Error bars represent standard deviation. [ANOVA results: $P<.001$ CNTF vs. 25K; $P<.01$ CNTF vs. NT-3; $P<.05$ 25K vs. 25K+CNTF, 25K vs. 5K]
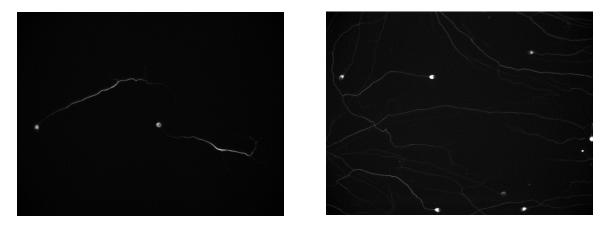
Figure 6. Representative image of SGNs in 25K (left) and CNTF+5K (right). Neurons in CNTF exhibit the longest neurites whereas neurons in 25K exhibit the shortest. SGNs are stained against NF200

in 5K is that they sustain their survival through autocrine signaling mechanisms (Hansen et al., 2001a, 2001b).This would explain their presence but it does not explain why they exhibit long neurites. As evident in figure 5, SGNs in 5K have longer neurites than those present in 5K+NT-3. In the end it may be difficult to directly compare neurites as it is possible that SGNs from these two conditions represent two distinct populations of neurons.

The Survival Effect of NTFs on Apical and Basal SGNs

SGNs along the tonotopic axis exhibit different morphological and electrophysiological phenotypes. The electrical phenotypes can be partially explained by the unique composition of voltage gated K^+-channels and Ca^{2+}-activated K^+ channels (Adamson et al., 2002; Chen & Davis, 2006). Furthermore, basal and apical neurons are exposed to different concentrations of NT-3 and BDNF (Schimmang, et al., 2003; Sugawara et al., 2007). Whether the electrophysiological difference is a consequence of the cochlear milieu or an inherent feature of the neurons is an issue being investigated. Nevertheless, these differences provoke the question whether apical and basal SGNs respond differently to CNTF.

Dissection of the ganglia was performed similarly as that used for figure 2. However, in this case the ganglion was divided into thirds using only the top and bottom thirds for experimentation. BDNF was included as a positive control. Unlike the survival results presented in figure 2 the results in figure 7 are more varied and inconsistent. BDNF and CNTF promote survival but CNTF was slightly less efficacious than that observed on the entire cochlea (see figure 2). No survival effect was observed for NT-3 in either the apical or basal condition. This was not expected and is believed to be due to technical issues. Regardless of the aberrant result the conclusion drawn is that there is no difference in survival between apical and basal derived neurons upon exposure to CNTF and BDNF.
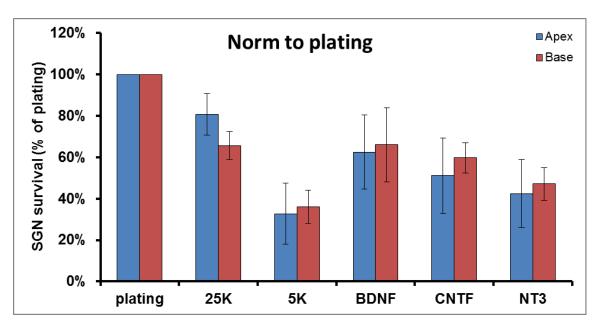
Figure 7. The survival effect of NTFs on apical and basal neurons. Survival is indexed as the percentage of the plating condition. N=5 biological repeats. Error bars represent standard deviation. [ANOVA Dunnet Multiple comparison test between conditions and respective control (5K): The following are significant. 5K apex vs. BDNF apex, 5K apex vs. CNTF apex, 5K base vs. BDNF base, 5K base vs. CNTF base]

<u>Neurite Effect of NTFs on Apical and Basal SGNs</u>

In addition to assaying survival, neurite lengths were measured from the conditions presented in figure 7. Unlike the variation observed for survival, the neurite lengths were more consistent from experiment to experiment. A graphical representation is given in figure 8. In addition, Table 1 lists the conditions that were significant after the computed ANOVA.

The results demonstrate that CNTF promotes robust neurite growth in both apical and basal neurons. Similar to the survival results presented in figure 5, there is no difference in neurite lengths between apical and basal neurons under the same NTF condition. The results demonstrate that CNTF is better able at promoting neurite growth than BDNF. As for NT-3, I am hesitant to draw any conclusions involving this neurotrophin as figure 5 did not produce the expected survival effect. Of further interest

is the observation that apical neurons in 25K produced significantly shorter neurites than basal neurons in 25K. One possible explanation is that CaMKII or calpain is regulated differently between these two populations of neurons under depolarizing conditions. Regardless of the mechanism, the finding has relevance for cochlear implants. If apical neurons are more greatly inhibited by electrical stimulation than their basal kin it would suggest that apical neurons are less likely to regrow towards the cochlear electrodes. This is somewhat unfortunate as most patients that receive implants have only apical neurons to spare. Of course this is purely speculative and further experiments are needed to reveal the underlying mechanism.
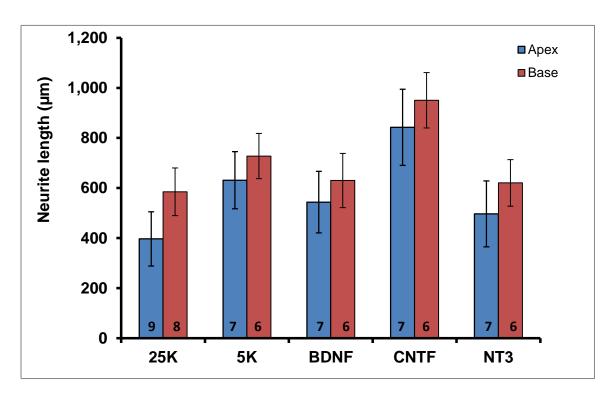
Figure 8. The neurite effect of NTFs on apical and basal derived neurons. Numbers in black represents the number of biological repeats. Error bars represent standard deviation.

Table 1. The significant ANOVA results presented in figure 8 are listed below.

Condition 1	Condition 2	Significance
25K (Apex)	25K (Base)	* P<0.05
25K (Apex)	5K (Apex)	** P<0.01
25K (Apex)	5K (Base)	*** P<0.001
25K (Apex)	BDNF (Base)	** P<0.01
25K (Apex)	CNTF (Apex)	*** P<0.001
25K (Apex)	CNTF (Base)	*** P<0.001
25K (Apex)	NT-3 (Base)	* P<0.05
25K (Base)	CNTF (Apex)	** P<0.01
25K (Base)	CNTF (Base)	*** P<0.001
5K (Apex)	CNTF (Base)	* P<0.05
5K (Apex)	CNTF (Base)	*** P<0.001
5K (Base)	CNTF (Base)	* P<0.05
BDNF (Apex)	CNTF (Apex)	*** P<0.001
BDNF (Apex)	CNTF (Base)	*** P<0.001
BDNF (Base)	CNTF (Apex)	* P<0.05
BDNF (Base)	CNTF (Base)	*** P<0.001
CNTF (Apex)	NT-3 (Apex)	*** P<0.001
CNTF (Apex)	NT-3 (Base)	* P<0.05
CNTF (Base)	NT-3 (Apex)	*** P<0.001
CNTF (Base)	NT-3 (Base)	*** P<0.001

CHAPTER 4

DISCUSSION

It has long been viewed that the survival of SGNs relies on neurotrophin support released from sensory cells. However, as reviewed in the introduction accumulating evidence is suggesting that SGNs can survive in the absence of hair cells. This undermines the importance of hair cells and implies that SGNs are receiving trophic support from non-sensory cells. Unfortunately, the exact location and nature of this source is currently unknown. This query has prompted others to perform microarrays and QT-PCR on cochlear tissue with the hope of uncovering the source and identity of these potential trophic factor(s) (Bailey et al., 2012). The results by Bailey (2012) have suggested that these novel factors may be CNTF, GDNF and NRTN arising from regions disparate as the organ of Corti and cochlear nucleus. Thus the purpose of this study was to ascertain whether these factors are trophic to spiral ganglion neurons. This hypothesis was tested by culturing postnatal day 5 SGNs in the presence of exogenous NTFs. The results demonstrate that CNTF promotes both survival and neurite growth of SGNs whereas GDNF and NRTN do not. In addition this study revealed that apical and basal neurons are equally responsive to CNTF both in regard to survival and neurite growth. During the course of this investigation CNTF was found to promote neurite growth more efficacious than BDNF and NT-3. Lastly, it was observed that apical neurons consistently produce shorter neurites than their basal counterparts under depolarizing conditions. The observation that CNTF promotes survival *in vitro* suggests that CNTF may be trophic to neurons *in vivo* and could explain why few SGNs remain present long after deafening.

The lack of effect with NRTN suggests that SGNs *in vivo* are not capable of responding to this particular trophic factor. However, it should be stressed that my SGNs were isolated from postantal day 5 rats and it is possible that SGNs from the immature

cochlea do not respond to NRTN. In accordance with this is the observation that embryonic day 16 chick cochlear ganglion explants do not respond to NRTN (Hashino et al., 1999). However, it still possible that the lack of response to NRTN reverses with age. Indeed the expression of GFRα2 is present in the mature spiral ganglia albeit low levels (Bailey et al., 2012)

The lack of effect of GDNF is in contrast with prior studies and was therefore unexpected. The pro-survival effect of GDNF on SGNs has been reported by a number of different groups using various experimental approaches (Ylikoski, et al., 1998; Yagi, et al., 2000; Kanzaki, et al., 2002). As a result of the discrepency this study followed up with a number of controls for GDNF. A dose response curve across a 30 fold range was carried out but still yielded negative results with respect to survival. In addition, a positive control was conducted by assaying GDNF on superior cervival ganglia (SCG). The findings demonstrate that GDNF does promote survival of SCG thus strengthening my conclusion that GDNF does not support the survival of postnatal day 5 SGNs. To the best of my knowledge the only study that assayed GDNF on SGNs in culture was that carried out by Ylikoski (1998). There are a few differences in methodolgy between the 1998 study and the current thesis. Examples include the following: the inclusion of serum, the concentration of GDNF, incubator conditions and methodology of identifiying SGNs. Despite theses differences in technique, I cannot reconcile the results.

It should be noted that the discrepancies with GDNF extend beyond the current study. For example, controversy in the literature exist regarding the expression of RET in SGNs. Studies using QT-PCR have demonstrated the expression of RET in SGNs (Stover et al., 2000, 2001) whereas others have failed to observe its presence (Ylikoski, et al., 1998; Hashino et al., 1999). However, this may be irrelevant since GDNF can signal independently of RET. Lastly the finding by Stankovic (2004) belies the pro-survival effect of GDNF. The massive loss of neurons caused by disrupted neuregulin-ErbB signaling was followed by a 250% increase in cochlear GDNF mRNA. Given the

inconsistencies in the literature and differences in methodology the lack of effect by

GDNF observed in the present study may not be as surprising as initially perceived.

CHAPTER 5

FUTURE EXPERIMENTS

This study demonstrates that CNTF can promote survival of SGNs *in vitro*. However, it is not evidence for its requirement *in vivo*. Direct evidence for its requirement in the mature cochlea is currently lacking. To test the hypothesis that mature SGNs are receiving trophic support by CNTF one needs to KO either the CNTF ligand or CNTFRα receptor after cochlear development. Ideally, one would like to restrict KO of the CNTF/CNTFRα to the cochlea so as to not affect other important organs or systems. In addition to KO CNTF/CNTFRα *in situ* against CNTF & CNTFRα mRNAs should be carried out. Microarray and QT-PCR experiments cannot inform the investigator which cells are expressing CNTF/CNTFRα. *In situ* data would identify these cells and augment the interpretation of future experiments. In regard to the KO CNTF/CNTFRα there are multiple ways in which this can be done. I will describe only one possible method.

The CRE-loxP system is commonly employed as a way to KO a specific gene *in vivo* (Sauer, 1987,1988). CRE is a recombinase initially discovered in bacterial phage P1. The CRE recognizes a pair of palindromic sequences on DNA called LoxP. Upon recognition the enzyme excises the intervening DNA sequence. For that reason, investigators place LoxP sites flanking their gene of interest. In addition to the presence of LoxP sites the recombinase gene must be harbored in the organism's genome. Ideally the CRE gene is inserted downstream of a promoter that is specifically active in the tissue or cell of interest. This is to reduce excision of the target gene in unwanted tissue/cells. Thus, when the tissue specific promoter becomes active it transcribes the CRE recombinase, after translation the enzyme recognizes the LoxP sites flanking the gene of interest and performs its excision.

There are some obstacles one must overcome with this method. First is the choice of the promoter. Tissue or cellular specific promoters may be unbeknownst to the investigator. Second is the temporal expression pattern of the promoter. Ideally the investigator would like to control when and where the excision takes place. The use of a tissue specific promoter may guarantee where excision occurs but it does not guarantee when the excision happens. However, there are some tricks that lend greater manipulative control over the CRE-LoxP system. One trick is use of the CRE-ER^T system (Feil et al., 1996). This is a modified form of the CRE recombinase. Specifically, CRE recombinase is fused to the ligand-binding region of the estrogen receptor. This chimera cannot bind estrogen but rather binds another molecule called tamoxifen. When CRE-ER^T is translated it remains present in the cytoplasm and only translocates to the nucleus upon exogenous application of tamoxifen. This allows the investigator to control when excision occurs, unfortunately tamoxifen can exert unwanted side effects in the organism.

Not surprisingly, the CRE-ER^T/LoxP system is not 100% efficient. A couple factors can account for the decreased efficiency. First, expression off the particular promoter may be weak. Secondly, if CRE is expressed at high levels it does not guarantee translocation into the nucleus upon addition of tamoxifen. And lastly, translocation of CRE into the nucleus may not result in complete excision of the target gene. One trick to overcome these obstacles is to place a reporter gene downstream of the target gene such as GFP. The reporter gene is not transcribed in the presence of the LoxP-flanked gene. Only after the gene of interest has been excised is the reporter gene transcribed. This method allows the investigator to determine whether excision has proceeded.

I suggest KO CNTF or CNTFRα utilizing the system described above. Unfortunately, and to the best of my knowledge, there are no known spiral ganglion specific promoters. This is not to imply there are no specific promoters within the cochlea. Indeed outer hair cells appear to transcribe presten, a gene unique amongst themselves (Zheng et al., 2000). An alternative is the use of a neuronal specific promoter.

Examples of promoters may include neuron-specific enolase (NSE), synapsin 1, VGLUT, voltage gated Na$^+$ channels, thymine, and neurogenin1. Some of the mentioned genes are involved in the developmental aspect of the cochlea and I am uncertain whether expression off these promoters remains active in the mature cochlea. Further research is required to find a suitable promoter.

The use of CRE/LoxP to abolish CNTF/CNTFRα signaling is certainly not the only method available. Examples of other techniques involve the use of adenovirus mediated siRNA. Viruses can infect cells with great selectivity and siRNA can be delivered to "knock down" specific genes. In addition, viruses can be modified so as to label the transfected cell allowing the investigator to determine whether and where transfection has taken place. However, successful infection of *all* cochlear neurons is an unrealistic feat. Another method is the infusion of a CNTFRα antagonist. In principle swamping the system with the antagonist would decrease normal CNTF signaling. To the best of my knowledge the only CNTFRα antagonist currently available is one developed by Stahl et al (1995). The antagonist is a chimera of the extracellular portion of the CNTFRα fused to the extracellular portion of the gp130 subunit.

CHAPTER 6

CONCLUDING REMARKS

The current thesis demonstrates that CNTF is sufficient to promote survival of SGNs *in vivo.* This observation would support the hypothesis that the neurons remaining after deafening are receiving trophic support via CNTF. Indeed the 2007 study by Alam et al demonstrated that the remaining neurons are receiving trophic support as evidenced by phospho-CREB staining. Of particular note is the observation that physiological levels of CNTF remain the same in the spiral ganglia before and after deafening (Bailey et al., 2012). Yet, despite CNTF's ability to promote survival of SGNs *in vitro*, physiological levels of CNTF *in vivo* do not appear sufficient to maintain survival of *all* neurons after deafening. One explanation is that SGNs *in vivo* require multiple trophic signals; no single endogenous NTF may be sufficient to maintain survival of *all* neurons. Furthermore, as neurons begin to die after deafening the ratio of CNTF to SGNs in the spiral ganglia begins to increase. Thus, the remaining neurons after deafening may be experiencing elevated concentrations of CNTF. This increased ratio may be sufficient to keep the remaining neurons alive. It remains to be seen whether the lack of CNTF signaling in SGNs exacerbates their death. As previously discussed testing this idea would require the use of transgenic mice lacking CNTF or CNTFRα in the mature cochlea.

The trophic and neurite promoting ability of CNTF has clear implications for CI research and or SGN therapy. Currently much effort of CI research is being focused on finding ways to keep neurons alive and promote their growth towards cochlear electrodes. In culture CNTF can strongly promote both. However, it remains to be seen whether this effect is maintained *in vivo*. Despite the clear implications of CNTF on SGNs we must not allow ourselves to be rash. Until we understand the signaling

mechanisms of this cytokine on both neuronal and non-neuronal cells we should refrain, or at the very least, exercise caution when applying this potent factor in the human cochlea. The consequences of exogenous CNTF may exceed the desired effects.

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