THE ROLE OF NEUROTROPHINS IN REGULATING SPIRAL GANGLION MORPHOLOGY

By

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A dissertation submitted to the

Graduate School- New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

Written under the direction of

Robin L. Davis

And approved by

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New Brunswick, New Jersey

OCTOBER, 2014

ABSTRACT OF THE DISSERTATION

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Many structural features of spiral ganglion neurons are specifically designed to carry out frequency-specific coding. Specific morphological properties correspond to tonotopic position along the cochlea contour, which include soma area and axon length. To examine how these features are regulated, we used various preparations. Thus, soma area is altered in a predictable manner corresponding to the frequency location of its peripheral target tissue. The sensory endorgan alters the axon characteristics of the spiral ganglion neurons. The regenerated axons have peripheral nervous system-like (PNS) and central nervous system-like (CNS) axon profiles that enable the examination of putative PNS/CNS axon length ratios. Similar to their *in vivo* patterns, the putative peripheral axons regenerated from low frequency neurons extend their processes a longer distance than high frequency neurons. This pattern is altered when the neuronal explants are paired with peripheral target tissue isolated from different cochlear regions.

To identify the regulatory factors that control these structural features, we investigated brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3). These neurotrophins control the ion channel composition that mediates the neuronal firing patterns (Adamson et al., 2002) and the synaptic protein levels (Flores-Otero et al., 2007) in spiral ganglion neurons. For example, the exposure to BDNF transformed neurons isolated from the apical or basal region phenotypic properties to resemble basal neurons. In contrast, the exposure to NT3 transformed the phenotypic properties of both apical and basal neurons to resemble apical neurons. We investigated whether soma area and axon length conforms to the same regulatory mechanism. In

the neuronal explant cultures, exposure to BDNF or NT3 had limited or no influence on cell size. Moreover, the effect of the peripheral target tissue on axon length is modulated by BDNF and NT3. However, both neurotrophins have a differential result on the axon length of spiral ganglion neurons isolated from distinct cochlear regions.

In all, frequency specific signals within the organ of Corti direct the structural phenotype of spiral ganglion neurons. These results indicate a different regulatory mechanism is needed to alter the structural phenotype within the spiral ganglion.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Robin Davis for her support and guidance throughout my graduate training. Thank you to my committee members Dr. Mark Plummer, Dr. Shu-Chan Hsu, Dr. Elizabeth Olson, and Dr. Cheryl Dreyfus for their mentorship during my graduate career. Thank you to Dr. Jacqueline Flores-Otero, Dr. Qing Liu, and Dr. Wei Chun Chen I am grateful for your friendship. I would like to thank the members of the laboratory Ms. Wenke Liu and Dr. Robert Crozier for your support and friendship. Mrs. Hui Zhong Xue, thank you for all the support and expert technical skills you given to the laboratory. Thank you to Mr. Zachary Wismer, Mr. Edmund Lee, and Mr. Shail Patel for their contribution to this project. Dr. Evelyn Erenrich, thank you for introducing me to the graduate program at Rutgers and your guidance. Dr. Nancy Woychik and Dr. Evelyn Erenrich thank you for helping me obtain a NIH post-baccalaureate fellowship. Dr. Jerome Langer, thank you for your helpful career advice.

Thank you to my parents, brother, and sister for your endless love and encouragement for me during my time in graduate school. I could have never achieved this much without your constant support. Mrs. Robin Mitchell and Mrs. Allison Smith-Dossou, Esq., thank you for your helpful advice and critique during the writing process. I would like to thank God for being with me throughout this journey.

Thank you to the IMSD/Bridge program. The NIH NIDCD R01 DC-01856 grant supported this work. A portion of this project is prepared in a manuscript form (Smith and Davis, in preparation).

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INTRODUCTION

Spiral ganglion neurons play an important role in transferring auditory information transduced by the hair cells to the central nervous system. Their morphology is different compared to most other neurons. They have a bipolar phenotype, which situates the somata within the action potential conduction pathway as a result the size of the soma and the diameter of the axon greatly impact action potential generation and conduction. Considerable research has been conducted characterizing the different structural properties of spiral ganglion neurons such as soma area, axon diameter, and axon length (Fekete et al., 1984; Liberman and Oliver, 1984; Echteler et al., 2000; Nadol et al., 1990; Rosbe et al., 1996). However, past studies have not focused on identifying the regulating factors mediating these different morphological features.

This present study examines the tonotopically-varying structural features of spiral ganglion neurons specifically soma area and axon length. We investigated these structural features under control conditions to examine the factors involved in their regulation and relate our findings to observations in vivo. In addition, we examined the impact of the hair cells and their surrounding supporting cells on altering these two structural parameters. Lastly, we examined critical factors involved in the regulation of these two phenotypic properties. These different morphological features of spiral ganglion neurons can impact the transmission of auditory information. Therefore, determining how soma area and axon length are regulated will enhance our understanding of auditory function.

The different Mechanical and Structural Properties within the Cochlea

Auditory information is processed by three elements within the ear which includes the following: the outer ear, the middle ear, and the inner ear. Sound information from the outside environment travels through the outer and middle ear. These sound waves are transformed into mechanical vibrations that are converted into fluid waves within the cochlea (the auditory portion of the inner ear). The cochlea breaks down complex sounds into their individual frequencies. The structural properties within the cochlea contribute to its ability to achieve precision in frequency discrimination. For example, the non-uniform shape of the basilar membrane is narrow and stiff at the basal end as well as wider and flexible at the apical end (Johnstone et al., 1986; Ren, 2002; Emadi et al., 2008; Naidu and Mountain, 1998). The uneven shape of the basilar membrane causes it to maximally vibrate at different positions depending on the stimulus frequency. The high frequency signals oscillate the basal end of the membrane and the low frequency signals oscillate the apical end, which gives rise to a topographical map of frequency. The hair cells are positioned on top of the basilar membrane (which runs along the length of the cochlea) in response to a stimulus frequency the hair cells move up and down. The hair cells have structural properties that differ with frequency region such as the stereocilia length, number, and width (Fuchs et al., 1988; Tilney and Saunder, 1983). At the apical end the stereocilia is longer, fewer in number, and narrower. At the basal end the stereocilia is shorter, wider, and more abundant (Tilney and Saunders, 1983). The cell body of the hair cells differs tonotopically; it is largest in the apex and shortest in the base (Fuchs et al., 1988). The ability of the cochlea to detect a wide range of frequencies is based on the different structural and mechanical properties of the hair cells and the basilar membrane that vary with cochlear location.

The hair cells convert the mechanical energy of the fluid wave into an electrical signal that is transmitted synaptically to the spiral ganglion neurons. The spiral ganglion neurons serve as the primary-sensory afferents, which transmit the electrical signals that are generated by the hair cell receptors to their central targets. However, these neurons have an unusual morphology because their soma area is located within the conduction pathway. The action potential is generated close to the receptor cell and is transmitted through the cell body to reach its central targets (Hossain

et al., 2005). As a consequence, the soma acts like a frequency filter and limit the passage of high frequency signals (Robertson, 1976). Surrounding the cell body are nodes of Ranvier that contain voltage gated sodium channels (Hossain et al., 2005; Fryatt et al., 2009). These voltage gated sodium channels increase the safety factor and decrease the chance of conduction failure due to axon - soma impedance mismatch (Luscher et al., 1994). This suggests a relationship exists between the morphological properties of a neuron and its functional characteristics.

The Basic Structural Organization of the Spiral Ganglion Neurons

The inner ear consists of the organ of Corti, a specialized organ involved in the reception of auditory stimuli. This organ consists of two sensory receptor cells that are aligned along the extent of the cochlea. The inner hair cells (IHCs) are arranged in a single row while the outer hair cells (OHCs) are arranged in three parallel rows. The hair cells send auditory information to the spiral ganglion neurons, which then transmits this information to the central nervous system. Spiral ganglion neurons consist of two populations the type I neurons, which innervate the IHCs and the small population of type II neurons, which innervate the OHCs. Type I and type II spiral ganglion neurons have different properties which include the following: hair cell innervation, soma size, abundance, and characteristic of its central and peripheral processes. Type II neurons innervate about thirty to sixty outer hair cells (Spoendlin, 1969,1972). Therefore, a small number of Type II neurons receive information from many OHCs. Type II spiral ganglion neurons (make up 5% of the population) have a small cell body and are bipolar or pseudomonopolar (Kiang et al., 1982; Berglund and Ryugo, 1987; Brown et al., 1988). The axons of Type II neurons are relatively thin and unmyelinated (Kiang et al., 1982; Romand and Romand, 1987; Brown, 1988). In contrast, Type I neurons make one-to-one synaptic connections to each inner hair cell (Ryugo, 1992). The IHC receives synaptic connections from ten to thirty spiral ganglion neurons (Liberman, 1980; Spoendlin, 1969, 1972). Type I neurons are bipolar and make up 95% of the population. A characteristic of the peripheral axon is its thinner in diameter in comparison to the central axon, which is observed across different species (Kiang et al., 1982; Berglund and Ryugo, 1987; Liberman and Oliver, 1984). The peripheral axon diameters of spiral ganglion neurons

have a slight tonotopic variation (Liberman and Oliver, 1984). The caliber, mitochondria content, and the location of the peripheral axon innervating the inner hair cell correlate to spontaneous rate (Liberman, 1982; Liberman and Oliver, 1984). A smaller axon diameter is associated with cells that fire at low or medium spontaneous rates and located on the modiolar side of the hair cell (Liberman and Oliver, 1984). A larger axon diameter is associated with cells that fire at high spontaneous rates and located on the pillar side of the hair cell (Liberman and Oliver, 1984). The larger diameter of the high spontaneous rate axon suggests it might have a faster conduction speed compared to medium or low spontaneous rate axons. A large axon diameter would increase action potential conduction speed and decrease internal resistance. However, it is uncertain whether the axon diameter distinctions between the low and high spontaneous rate neurons are regulated in the peripheral auditory system to vary conduction speeds (Liberman and Oliver, 1984). Another property that optimizes conduction time is myelination. While, the axons are ensheathed in compact myelin the large cell body of Type I neurons is surrounded by loose myelin (Rosenbuth et al., 1962; Romand and Romand, 1987). A second suggestion to explain the axon diameter differences between medium or low and high spontaneous rate axons is activity level. The high spontaneous rate axons are generally higher in mitochondria compared to low or medium spontaneous rate axons (Liberman et al., 1980). Therefore, the increase in metabolic activity may associate with the higher spike activity levels from a high spontaneous rate neuron (Liberman et al., 1980). The only part of the neuron that does not correlate to spontaneous rate is the cell body.

Soma area has been well characterized across different species such as cats, gerbils, and humans. Specifically, this project examined how soma area gradients are controlled. The structural analysis of spiral ganglion neurons in cats is the most complete with data that correlates physiological and structural findings (Liberman and Oliver, 1984). Liberman and Oliver used horseradish peroxidase to label axon fibers, which then could be reconstructed from their peripheral ending on the cochlear hair cells to the region the processes enter the cochlear nucleus. The benefit of this procedure is an unequivocal reconstruction of a single fiber trajectory. All of the intracellularly labeled neurons are type I cells innervating IHCs. The analysis of the cell

body shows soma area varies with cochlear location. Neurons with the largest cell size are located in the low frequency region while neurons in the high frequency region have a smaller soma area. This finding is opposite when compared to Leake and colleagues analysis of soma area in cats (Leake et al., 2011). The cross-sectional area of the cell somata in four normal adults cats show cell size is largest in the base and decreases towards the apex. This study reiterates apical and basal neurons soma area measurements in gerbils and humans. The small sample population measured and the heterogeneity in apical and basal neurons soma area may account for the size differences observed in the Liberman and Oliver paper. Soma area analysis from adult human tissue is evaluated in serial sections (Rosbe et al., 1996; Nadol et al., 1990). The limitation using human tissue is heterogeneous genetic background and the incomplete background on noise or ototoxic exposure, which may reflect an unknown phenotype. The human tissue in the analysis of soma area is selected from regions with normal pathology. The cross-sectional area of the cell body is largest in the basal turn, the intermediate cells are in the middle turn, and the smallest cells are in the upper middle turn.

The soma area of gerbils is examined in the postnatal cochlea (Echteler et al., 2000). The cross-sectional area of the somata is examined in the basal, middle, and apical regions. The cell size reiterates soma area measurements previously observed in adult animals. Echteler and colleagues suggest the regional difference in cell growth is due to the size of the Rosenthal's canal. The concentration of the cells within each region influences the growth due to the availability of extracellular space. This present study does not support this conclusion to explain the regional differences in the size of spiral ganglion neurons. We utilize different *in vitro* preparations to examine cell size of spiral ganglion neurons isolated from the extreme apical and basal regions of the cochlea. The experiments carried out herein show the peripheral target tissue by the factors they secrete can influence soma area regulation of spiral ganglion neurons.

The experiments that are implemented within this study reiterate the same soma area differences in vivo (between apical and basal neurons). The goal is to investigate the control mechanism that mediates the graded soma size along the ganglion that is specifically to designed to regulate signal processing in the neurons. Therefore, we can utilize this experimental system

to examine soma area and to determine the factors that are involved in its regulation.

i. Axonal Outgrowth Patterns of Spiral Ganglion Neurons

The tonotopic relationship for soma area in spiral ganglion neurons is also evident in other structural features such as axon length. The original studies evaluating the structure of individual neurons used the Golgi technique but this method works best in neonatal and younger animals (Lorente de No, 1933; Brawer and Morest, 1975; Feldman and Harrison, 1969). The axon projection length was examined in adult cats using the intracellular recording technique to stain the axon fibers with horseradish peroxidase. This technique characterizes the distinct anatomical features and electrophysiological properties of the auditory nerve fibers. The peripheral axon extends from the organ of Corti to the internal auditory meatus (IAM) (Fekete et al., 1984). The central axon extends from the IAM to the bifurcation (Fekete et al., 1984). The axon bifurcation establishes the ascending and descending branches which innervates synaptic targets in the cochlear nucleus (Ryugo, 1992; Ryugo et al., 1988; Ryugo and Fekete et al., 1984). Also synaptic specializations are localized in the axonal fibers near the IAM (Brown et al., 1988). This region has the potential to be used as a marker between the peripheral nervous system (PNS) /central nervous system (CNS). Based on the cochlear location the tonotopic difference arises in the proportions between the peripheral and central axon lengths. In the peripheral region, apical neurons are farther from the IAM and must project their axons a longer distance (Liberman and Oliver, 1984). In contrast, basal neurons are closer to the IAM and project their axons a shorter distance. In the central region, after crossing the IAM, apical neurons immediately bifurcate; while the axon of basal neurons extend a longer distance before it bifurcates (Fekete et al., 1984). However, the length of the projecting axon from the organ of Corti to its central synapse is the same for apical and basal neurons (Fekete et al., 1984). Overall, it is the proportion of the PNS/ CNS axon lengths that differ between apical and basal neurons.

This present study shows the axons regenerate PNS-like and CNS-like characteristics in culture. We characterize this morphological phenotype using a specialized culture bioassay developed in the Davis Lab, termed the synapse culture. This culture consists of regions of the

organ of Corti containing the inner and outer hair cells along with their surrounding satellite cells (micro-isolates) and placed near a spiral ganglion neuronal explant. By separating the tissue in this manner we are able to co-culture the hair cell micro-isolates from different frequency regions with spiral ganglion neuronal explants isolated from the apex or base. Both structural and biological characteristics are utilized to describe the differences between the two axon profiles. Evaluating the axon ratio profiles under control conditions, we can examine the underlying mechanism involved in mediating this morphological phenotype.

In conclusion, spiral ganglion neurons have different morphological features, which include the diameter of the axon, the size of the soma, and the axonal outgrowth. This project particularly is focused on the latter two structural features that are graded tonotopically. In addition, this study examines the involvement of neurotrophins in the regulation of spiral ganglion morphology. In order to better understand neurotrophins involvement in mediating these parameters, a general overview of neurotrophins must be addressed.

Neurotrophins and their Receptors distribution within the cochlea

Little is known about the mechanism that regulates soma area and axon length which is systematically graded along the cochlear contour. The present study shows the hair cell micro-isolates can influence soma area and axon length of spiral ganglion neurons. The neurotrophins are the first candidates investigated to identify the regulatory molecules mediating spiral ganglion morphology. This study establishes the role that the hair cells and their surrounding satellite cells via neurotrophic factors play in regulating these phenotypic properties.

Neurotrophins are structural related proteins that regulate survival, differentiation, and synaptic plasticity of neurons throughout the peripheral and central nervous system (McAllister et al., 1997; Pang et al., 2004). They are synthesized as proneurotrophins, which are cleaved in the endoplasmic reticulum and converted to the mature form; or the unprocessed form is transported to the plasma membrane and released (Mowla et al., 2001;Suter et al., 1991). In this situation, the protein is cleaved by plasmin or another extracellular protease, which converts the precursor protein to the mature neurotrophin through proteolytic cleavage (Lee et al., 2001;Nagappan et

al., 2009). The best characterized family of growth factors are neurotrophins which includes the nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5). In the development of the cochlea, BDNF and NT3 are the prominent factors that support spiral ganglion neuronal survival and maintenance (Farinas et al., 2001; Pirvola et al., 1994,1992; Ylikoski et al., 1993).

In situ studies of the inner ear show BDNF and NT3 are expressed by the sensory epithelia and the sensory neurons express their specific Trk (tropomyosin-related kinase) receptor belonging to the family of tyrosine kinases (Pirvola et al., 1992, 1994; Schecterson and Bothwell, 1994; Wheeler et al., 1994). The trkB receptor specifically binds to BDNF and NT4/5 as well as NT3, but at a lower affinity. The trkC receptor specifically binds to NT3 (Klein et al., 1991; Klein et al., 1992; Davies et al., 1995; Lamballe et al., 1991). Vigers and colleagues demonstrate the lacZ reporter is more sensitive than the in situ hybridization technique. The coding region for NT3 or BDNF is replaced by lacZ, which is the gene that codes for the enzyme β-galactosidase. The histochemical detection of β-galactosidase has a higher signal to noise ratio and a sensitive method to examine the expression pattern of proteins (Vigers et al., 2000). Farinas and colleagues examine the expression pattern of BDNF and NT3 using lacZ inserted into the NT3 and BDNF loci. The expression of BDNF at E 16.5 and above in the developing cochlea extends in an apical-to-basal pattern in the hair cells but the expression of BDNF is larger in the apical region (Farinas et al., 2001). During embryonic development, NT3 expression develops in a base to apex progression. The expression of NT3 follows the maturational gradient of the cochlea (Zine and Romand, 1996; Kaltenbach and Falzarano, 1994). NT3 expression is mainly in the supporting cells such as the deiter's cells, pillar cells, and Border cells (Pirvola et al., 1992; Farinas et al., 2001).

The targeted gene disruption of a single neurotrophin or receptor causes a specific loss of cochlear neurons from distinct regions of the cochlea. Three independently generated NT3 null mutant mice with different genetic backgrounds demonstrate a uniform phenotype. The mutant mice have a complete loss of neurons in the basal turn and the absence of radial fibers to the sensory epithelium within this same region (Ernfors et al., 1995; Fritzsch et al., 1997; Coppola et

al., 2001;Liebl et al., 1997). The lipophilic dye DiI (1,1'-diotadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate) labeling is a tracing technique used to examine afferent and efferent fibers in the NT3 null mutant mice (Fritzsch et al., 1997; Coppola et al., 2001;Liebl et al., 1997). The neuronal loss in the NT3 null mutant is mainly in the basal turn but a number of neurons are still present within the middle and apical regions (Farinas et al., 2001). Farinas and colleagues conclude that the NT3 null mutant has no neurotrophin to support the neurons in the basal turn. This is because BDNF is not expressed in the basal turn epithelium during this time period. This view is supported by the longitudinal expression gradient of BDNF in the hair cells, which matches the maturation gradient of the cochlea developing from apex to base.

A similar result is demonstrated in studies with the trkC kinase-deficient mice (Schimmang et al., 1995; Schimmang et al., 1997). The same tracing technique is used in the NT3 null mutants studies shows a loss of basal turn sensory neurons (Fritzsch et al., 1998). The neuronal loss is not as severe when compared to the NT3 null mutant result. The trkC-full length null mutation (Tessarollo et al., 1997) has a very similar result observed in the NT3 null mutant studies a loss of basal turn sensory neurons (Fritzsch et al., 1997; Coppola et al., 2001;Liebl et al., 1997). The different result in the ligand and receptor knockout studies may suggest the truncated non-catalytic isoform of trkC receptors is present. In the Schimmang and colleagues studies, this receptor may either sequester the ligand or interact with other full length trk receptors such as trkB to support the limited population of sensory neurons (Schimmang et al., 1995; Schimmang et al., 1997).

The trk receptor null mutation result is confirmed in other mutant mice studies. The first study examines the bHLH (basic helix-loop-helix) transcription factor Neuro D. In NeuroD-null mice, the expression of trkC in the sensory neurons is reduced leading to the specific loss of neurons in the basal region (Kim et al., 2001). The second study examines the POU domain transcription factor Brn3a which mediates survival, soma size, axon pathfinding and migration (McEvilly et al., 1996;Huang et al., 2001). The loss of Brn3a causes the downregulation of trkC expression in spiral ganglion leading to a similar innervation defect observed in the trkC receptor deficient mice (Huang et al., 2001). The results from both studies are similar to observations in the trkC

receptor null mutant mice. Overall, the results support the conclusion of NT3 supporting the neurons in the basal region through the trkC during embryonic development.

The absence of BDNF resulted in the largest reduction of neurons and their projections in the apical region (Bianchi et al., 1996). This result is confirmed by Farinas and colleagues showing a specific loss of sensory neurons in the apical region using the BDNF null mutant mice. They suggest in the BDNF null mutant the loss of apical neurons is due to the insufficient expression of NT3 at this early stage to support survival (Farinas et al., 2001). This is supported by the expression gradient of NT3, which matches the maturation gradient of cochlea developing from base to apex (Farinas et al., 2001). The trkB receptor null mutation has the most pronounced reduction of apical and middle sensory neurons and their innervations (Fritzsch et al., 1998).

The studies examining NT3, BDNF, trkB-kinase, and trkC-kinase null mutant mice suggest that spiral ganglion neurons have a regional specific dependence on a particular neurotrophin. The spiral ganglion neurons in the basal region are dependent on NT3/trkC. The neurons in the middle and apical regions are dependent on BDNF/trkB during the embryonic development of the cochlea.

In the postnatal and mature cochlea, the distribution pattern is the opposite of the embryonic pattern. The tissue preparations utilized in this present study are isolated from the postnatal cochlea. The description of the expression pattern of BDNF and NT3 is important to the role these neurotrophins play in regulating the morphological features of spiral ganglion neurons that changes with tonotopic location. In the adult cochlea, in situ hybridization studies report NT3 is expressed by the IHCs (Pirvola et al., 1994; Wheeler et al., 1994; Ylikoski et al., 1993). In the early postnatal period, NT3 expression is observed in the supporting cells (Qun et al., 1999;Pirvola et al., 1992). The Stankovic and colleagues examined the cochlea of mice in which the *Escherichia coli* lacZ gene is integrated into the NT3 locus to examine NT3 expression in the postnatal day (P) 26 cochlea. The histological analysis of LacZ staining shows NT3 is most prominent in the IHCs and their supporting cells (Stankovic et al., 2004).

In the postnatal inner ear, the pattern of NT3 expression is examined using the NT3-lacZ reporter mouse (Sugawara et al., 2007). This study examines NT3 expression at different

postnatal age groups. They report NT3 expression is highest in the apex and lowest in the base in all ages from P0-P135. The expression levels of NT3 remained high well into adulthood (Sugawara et al., 2007). Findings from the Davis laboratory show the region specific expression of NT3 plays a role in specifying the electrophysiological features in spiral ganglion neurons predominantly present in the apical region. For example, when NT3 is exogenously applied to postnatal spiral ganglion neurons it alters the electrophysiological and synaptic protein phenotypic properties to resemble an apical characteristic.

Studies examining BDNF expression pattern in the postnatal and mature cochlea have conflicting results. In situ hybridization studies show, in the postnatal cochlear tissue, BDNF mRNA expression is either nonexistent in the organ of Corti (Wheeler et al., 1994) or detected at low levels (Pirvola et al., 1992; Ylikoski et al., 1993). In the adult cochlea, BDNF is not expressed in the organ of Corti (Wheeler et al., 1994; Ylikoski et al., 1993). Wiechers and colleagues evaluated the expression patterns of BDNF at different postnatal age groups from P1, P3, P6, and P10. They found the expression of BDNF mRNA and protein levels are upregulated and downregulated in the organ of Corti (Wiechers et al., 1999). The location of BDNF protein in the organ of Corti is analyzed using immunohistochemistry. The examination of the organ of Corti shows at P1 BDNF is expressed, P3 BDNF is absent, P6 BDNF is present, and at P10 is downregulated (Wiechers et al., 1999). This pattern of expression could explain the conflicting results reported in the less comprehensive earlier analysis of BDNF expression in the postnatal cochlea. In postnatal tissue sections, Flores-Otero and Davis show BDNF is distributed relatively equally between inner and outer hair cells. The immunolabeling of BDNF is greater in the basal than apical hair cells (Flores-Otero and Davis, 2011). In the postnatal cochlea, BDNF mRNA is expressed in the spiral ganglion neurons (Wiecher et al., 1999;Hansen et al., 2001;Singer et al, 2008; Ruttiger et al., 2007; Schimmang et al., 2003). Some studies show a gradient of BDNF mRNA expression highest in the basal compared to the apical region (Schimmang et al., 2003; Ruttiger et al., 2007). The expression pattern of BDNF in the postnatal and adult cochlea plays an important role in regulating region specific characteristics of spiral ganglion neurons. Findings from the Davis laboratory show the region specific expression of BDNF plays a role in specifying

the electrophysiological features in spiral ganglion neurons predominantly in the basal region. For example, when BDNF is exogenously applied to postnatal spiral ganglion neurons it alters the electrophysiological and synaptic protein phenotype to resemble a more basal characteristic.

In summary, NT3 concentration level is highest in the low frequency compared to the high frequency region in the cochlea (Fritzsch et al., 1997; Pirvola et al., 1992; Sugawara et al., 2007; Ylikoski et al., 1993. At high concentrations, NT3 has the ability to bind multiple receptors (trkB and or/ the p75 neurotrophin receptor). As a result, NT3 can have multiple responses on the phenotype of spiral ganglion neurons. Whether BDNF is produced and secreted by the hair cells is unresolved during postnatal development. Wiecher and colleagues show BDNF expression is upregulated and downregulated throughout different postnatal time periods (Wiecher et al., 1999). This pattern of expression can explain why BDNF presence at this developmental time period in the cochlea is unclear. The presence of BDNF predominantly in basal spiral ganglion neurons (Schimmang et al., 2003; Ruttiger et al., 2007) suggests BDNF may have an autocrine role in the phenotypic regulation of spiral ganglion neurons.

Other neurotrophic factors in the cochlea examined during auditory development. The neurotrophin NT4, also known as NT5, Pirvola and colleagues show using in situ hybridization no NT4 mRNA in the rat organ of Corti (Pirvola et al., 1992). In the early postnatal and adult rat cochlea, NT4 mRNA is not detected in any inner ear structures (Ylikoski et al., 1993). Bianchi and colleagues using the NT4 null mutant mice show the cochlear neurons did not reduce throughout development (Bianchi et al., 1996). This result is consistent with the studies indicating NT4 is not detected in the inner ear tissue (Pirvola et al., 1992; Ylikoski et al., 1993). However, Zheng and colleagues report NT4 promoted the survival of postnatal rat spiral ganglion neurons in dissociated cell cultures (Zheng et al., 1995). Moreover, Mou and colleagues report spiral ganglion neuronal survival is enhanced when exposed to different concentrations of NT4 (Mou et al., 1997). NT4 is not expressed in the peripheral auditory system but it can still have an application in vitro since it works through the trkB receptor. Glial cell line-derived neurotrophic factor (GDNF) expression at postnatal day 7 is present in the hair cells (Ylikoski et al., 1998). However, its receptor RET (the transducing receptor of GDNF) is not present in the guinea pig

cochlea during early development (Ylikoski et al., 1998). However, it is observed at low levels in spiral ganglion cells (Stover et al., 2000; Kuang et al., 1999). Ciliary neurotrophic factor (CNTF) is present in spiral ganglion neurons. The receptor, CNTFa, is expressed in the organ of Corti (Malgrange et al., 1996). This analysis is confirmed with microarray gene expression that CNTF and CNTF receptors are expressed in the ganglion (Bailey et al., 2012). BDNF and NT3 are not the only neurotrophic factors expressed in the cochlea that has the potential to mediate different phenotypic aspects of spiral ganglion neurons.

i. Distribution of neurotrophins receptors within the cochlea

The survival of spiral ganglion neurons is mediated by either BDNF or NT3 (Mou et al., 1997; Malgrange et al., 1996; Hegarty et al., 1997; Lefebvre et al., 1994) through binding to its specific Trk receptor. All neurotrophins bind with the same affinity to the p75 neurotrophin receptor ($p75^{NTR}$) which is a member of the tumor necrosis factor receptor family (Bibel et al., 1999). The $p75^{NTR}$ distribution pattern in spiral ganglion neurons is reported to change depending on developmental stage or auditory injury. Von Bartheld and colleagues used in situ hybridization to examine the mRNA expression of $p75^{NTR}$ in early spiral ganglion neurons (Von Bartheld et al., 1991.) In the rat embryos, the expression of $p75^{NTR}$ is localized to the spiral ganglion neurons. In the adult rats, $p75^{NTR}$ immunoreactivity is not detected in the cell bodies of the spiral ganglion neurons (Von Bartheld et al., 1991.) In the postnatal cochlea, Sato and colleagues show $p75^{NTR}$ immunoreactivity in the spiral ganglion neurons (Sato et al., 2006). They also examine whole-mount mice cochlea at 1month of age and the $p75^{NTR}$ is expressed in the neurons (Sato et al., 2006). Tan and Shepherd, in adult rats, report $p75^{NTR}$ expression is not in the spiral ganglion neurons but is detected at low levels in the peripheral processes in normal hearing animals. However, in the deafened cochlea the level of the $p75^{NTR}$ immunoreactivity is prominent in the peripheral processes and the Schwann cells. The intensity level of $p75^{NTR}$ in the degenerating peripheral processes indicates a pro-apoptotic response (Tan and Shepherd, 2006). Provenzano and colleagues confirm the previous report (Tan and Shepherd, 2006) demonstrating the enhancement of $p75^{NTR}$ expression after deafening in spiral ganglion Schwann cells (Provenzano

et al., 2011). The evaluation of the $p75^{NTR}$ immunoreactivity in the cochlear sections show staining is prominent in the Schwann cells and not the neurons (Provenzano et al., 2011). Several studies describe $p75^{NTR}$ is predominantly absent in the spiral ganglion neurons. However, $p75^{NTR}$ may have a protective role in the cochlea in response to trauma. The $p75^{NTR}$ expression is elevated in the mature cochlea due to an injury and up-regulated in non-neuronal cells. This leads to a reduction in the loss of spiral ganglion neurons. The mechanism is not known, but Provenzano and colleagues suggest after deafening the $p75^{NTR}$ transmembrane domain is cleaved resulting in the release of the intracellular domain (ICD) (Provenzano et al., 2011). The ICD is then translocated to the nucleus and regulates spiral ganglion Schwann cell proliferation. The $p75^{NTR}$ indirectly has a protective response by maintaining the Schwann cell population. The Schwann cells produce neurotrophins such as NT3 (Hansen et al., 2001) providing trophic support to the spiral ganglion neurons.

The $p75^{NTR}$ and the Trk receptor mediate different biological outcomes. Following ligand binding, the Trk receptors dimerize (Jing et al., 1992). This leads to the phosphorylation of specific tyrosine residues located in the activation loop of the tyrosine kinase domain (Cunningham and Greene, 1998). As a result, the receptor changes its conformation resulting in trans-phosphorylation enabling the access of substrates to the kinase. The phosphorylated tyrosine residues on the Trk receptors act as docking sites for adaptor molecules. The two tyrosine residues are: the NPXY mortiff in the juxtamembrane domain (Stephen et al., 1994) and the YLDIG motif in the carboxy terminus (Obermeier et al., 1994). When the NPXY domain is phosphorylated this recruits other signaling molecules. The Shc molecule binds to the NPXY domain by its phosphotyrosine domain (PTB) (Obermeier et al., 1994; Obermeier et al., 1993;Stephen et al., 1994; Songyang et al., 1995). The Grb2 protein links to the Shc molecule and recruits Sos (guanine nucleotide exchange factor) to the membrane. This leads to the up-regulation of Ras, when bound to GTP, which promotes the activation of the MAP kinase cascade: Raf, MEK (mitogen-activated protein kinase/ERK kinase), and ERK (extracellular signal-regulated kinase). The phosphorylation of ERK leads to the activation of transcriptional events such as cyclic AMP-response element binding (CREB) transcription factor. CREB impacts the

differentiation of neurons, neurite growth, and synaptic plasticity (Lonze et al., 2002).

Another major signaling pathway is the PI3 kinase pathway. The phosphorylation at the NPXY domain recruits the Shc and Grb2. This complex recruits the adaptor molecule Gab-1 (Grb-associated binder) which mediates the PI3 kinase association to the Trk receptor (Holgado-Madruga et al., 1997). The PI3 kinase binds to Gab-1 and activates the catalytic activity of Akt kinase. This pathway is important in mediating survival due to the activation of the Akt protein kinase (Crowder and Freeman, 1998; Burgering and Coffer, 1995). This applies to spiral ganglion neurons the inhibition of Akt prevents BDNF and NT3 from promoting survival (Hansen et al., 2001). The third major pathway induced by Trk receptor activation is the PLC pathway. The phosphorylation of the YLDIG domain recruits PLC to the Trk receptor (Poole et al., 2004). This enzyme breaks down the lipids diacyl glycerol and inositol (1,4,5) triphosphate (IP3). The binding of IP3 to specific receptors regulates intracellular calcium levels. The diacyl glycerol induces activation of protein kinase C (PKC). Lallemend and colleagues demonstrate PKC activation induces neuroprotection of spiral ganglion neurons after an injury. In situ studies show PKCβ1is expressed in the postnatal and adult spiral ganglion neurons. The activation of PKCβ1 by PKC activators such as phorbolesters promotes spiral ganglion survival and neurite outgrowth (Lallemend et al., 2005). The biological activity of the Trk receptors has various outcomes such as neuronal survival, differentiation, and neurite outgrowth.

The cellular actions of $p75^{NTR}$ are dependent on its receptor complex formations. The $p75^{NTR}$ function is not necessarily proapoptotic (Teng et al., 2010). It can constrain axon growth (Teng et al., 2010) and down-regulate synaptic activity (Yang et al., 2009). The $p75^{NTR}$ does not have an intrinsic catalytic activity. As a result, this receptor signals by associating with or dissociating from different cytoplasmic interactors. The first adaptor proteins is the ubiquitously expressed zinc finger protein designated NRIF (neurotrophin receptor-interacting factor). Casademunt and colleagues show in retinal cells *nrif-/-* mice a reduction in cell death. This result is similar to the *p75-/-* findings suggesting that NRIF is a downstream component of $p75^{NTR}$ in the cell death pathway (Casademunt et al., 1999). A second interactor of $p75^{NTR}$ is NRAGE homolog (neurotrophin receptor-interacting MAGE). NRAGE is shown to regulate the NGF-dependent

apoptosis and cell cycle progression in mammalian cells (Salehi et al., 2000). A third interactor of $p75^{NTR}$ is the protein NADE (p75 NTR-associated cell death executor). NADE / p75 receptor induce cell death is dependent on NGF but not BDNF, NT3, and NT4 (Mukai et al., 2000). Other intracellular molecules that interact with the $p75^{NTR}$ is RhoA which regulates axon growth (Yamashita et al., 1999) and NF-κB, which is involved in enhancing neuronal survival in trigeminal neurons (Hamanoue et al., 1999). The $p75^{NTR}$ can interact with the three Trk receptors. When the $p75^{NTR}$ associates with the Trk receptor, it results in increasing the affinity of the neurotrophin to its specific Trk receptor (Bibel et al., 1999; Lee et al., 1994). Another binding partner is the type 1 receptor Sortilin which is a member of the family of Vps10p-domian receptors (Skeldal et al., 2012). The $p75^{NTR}$ becomes a high affinity receptor for proneurotrophins when associated with the Sortilin receptor (Teng and Hempstead, 2004). The receptor complex of $p75^{NTR}$ and Sortilin induces an apoptotic response (Lee et al., 2001; Nykjaer et al., 2004; Teng et al., 2005).

In the present study, both neurotrophins have the capability of having the same phenotypic response within a specific frequency region. For example, BDNF and NT3 is involved in increasing or decreasing axon length of basal or apical neurons. The similar response of both neurotrophins may indicate the p75 neurotrophin receptor is involved in the regulation.

ii. BDNF and NT3 regulates soma area

Neurotrophins alters the soma area of spiral ganglion neurons in addition to other types of neurons. The infusion of BDNF into the deafened cochlea *in vivo* increases basal soma area in comparison to normal controls (Shepherd et al., 2005,2008; McGuinness and Shepherd, 2005; Agterberg et al., 2008). McGuinness and Shepherd examine the soma area of spiral ganglion neurons in the deafened rats cochleae via ototoxic drugs following chronic delivery of exogenous BDNF. The deafening technique results in the complete loss of hair cells throughout all cochlear turns. The size of the spiral ganglion neurons in the BDNF treated cochleae is larger and more numerous when compared to the neurons in the normal hearing cochleae (McGuinness and Shepherd, 2005). Shepherd and colleagues examine the soma area of spiral ganglion neurons in

the deafened guinea pigs cochleae via intracochlear kanamycin infusion following exogenous delivery of BDNF. They report a similar observation; the soma area of basal spiral ganglion neurons treated with exogenous BDNF is similar to or greater than normal hearing controls. The soma area of apical neurons treated with BDNF is similar to normal hearing controls (Shepherd et al., 2005,2008). Agterberg and colleagues examine the soma area of spiral ganglion neurons in the deafened guinea pigs cochleae following treatment of BDNF. They report the basal soma area of spiral ganglion neurons after BDNF treatment is significantly greater than normal hearing controls (Agterberg et al., 2008). Leake and colleagues report soma area is comparable to normal controls. The neurons only within the basal region are slightly larger in the deafened cochleae of cats after intracochlear infusion of BDNF (Leake et al., 2011).

In the deafened cochleae, neurons in the basal region are predominantly rescued from degeneration following neurotrophin treatment (Shah et al., 1995; Wise et al., 2005; Rejali et al., 2007;Agterberg et al., 2008; Shepherd et al., 2005). The delivery site of the neurotrophin is either the round window or cochleostomy, but no connection is observed with respect to the type of neurotrophin or the delivery method to indicate the reason the basal region has the highest response. The easiest explanation for BDNF having the greatest influence on basal neurons is the delivery site location within the basal region. However, Miller and colleagues report a concentration of 50ng/ml BDNF is enough to enhance the survival of neurons at the base of the cochlea (Miller et al., 1997). This suggests only a small concentration is needed to enhance the survival of spiral ganglion neurons. Richardson and colleagues results demonstrate a single infusion of neurotrophin originating from the basal region is able to reach the apical turn of the cochlea (Richardson et al., 2004). This suggests that a low concentration of neurotrophin can diffuse into the apical region. Agterberg and colleagues suggest their larger dose of 100ug/ml of BDNF should reach the apex with the delivery site at the basal cochlear turn. In all, the influence of BDNF only in the basal turn is not due to BDNF being only localized in this region because the apical neurons also have access to this neurotrophin.

The issues that are debated within these studies are the following: is the increase in soma area due to BDNF, is the increase in size due to the larger concentration of BDNF mainly in the

basal region, or is other potential endogenous trophic factors interacting with BDNF in the deafened cochlea influencing cell size. This present study solves these different issues in the *in vivo* studies by using tissue culture. In the in vivo studies, BDNF response of increasing the soma area of basal neurons could not be certain because of the different issues previously described. However, tissue culture is a more controlled environment. We are able to treat each neuronal group the same. In culture, the apical and basal neuronal explants are isolated from their peripheral and central target tissue. Therefore, we can directly examine the response of BDNF or NT3 has on regulating soma area.

In addition, spiral ganglion neurons are not the only type of neuron to alter its soma size following BDNF treatment. The soma area of dopaminergic neurons is altered in the vertebrate retina after intraocular injections of BDNF. The analysis of whole-mounted tyrosine hydroxylase immunolabeled retinae show the dopaminergic cells increase in size when compared to controls (Cellerino et al., 1998). In hippocampal neurons, BDNF is reported to enhance the synthesis of K+-elected release of GABA, which increases the glutamic acid decarboxylase, a GABA-synthesizing enzyme and enlarged the soma area (Yamada et al., 2002). Lom and Cohen-Cory used microspheres treated with BDNF that is injected into the eye of live tadpoles to alter retinal neurotrophin levels. They report the exogenous application of BDNF to *Xenopus* retinal ganglion cells did not alter soma area when compared to control treated retina ganglion cells (Lom and Cohen-Cory, 1999). Perez-Navaao and colleagues report striatal projection neurons with BDNF treatment significantly increase cell size in comparison to untreated controls (Perez-Navaao et al., 1999). These results show the response of BDNF on soma area is not restricted only to spiral ganglion neurons but it can increase the soma area in many other cell types.

NT3 has been shown to both increase and decrease the soma size of neurons in different systems. Limited studies report NT3 decreasing cell size. Lom and Cohen-Cory report retina ganglion cells with NT3 treatment significantly decrease cell size in comparison to untreated controls (Lom and Cohen-Cory et al., 1999). NT3 has been shown to increase soma area. In deafened guinea pigs, spiral ganglion neurons after treatment with a single pulsed infusion of NT3 increase soma area to levels comparable to normal hearing controls (Richardson et al.,

2005). They report the continuous infusion of NT3 soma size is enhanced above normal hearing controls (Richardson et al., 2005). In both situations, NT3 enhanced cell size in spiral ganglion neurons. The soma area of dorsal root ganglion neurons at E16 increase cell size following the in utero injection of NT3 compared to control (Zhang et al., 1994). The soma area of cultured embryonic striatal neurons with NT3 treatment increases cell size compared to control (Nakao et al., 1996). In the spiral ganglion, NT3 is capable of having a bidirectional outcome on soma area and influences size of different types of neurons.

In this present study, we investigated using culture preparations the response of BDNF and NT3 on the soma area of spiral ganglion neurons. Overall, this study explores the hypothesis that the large size of basal neurons is regulated by BDNF and the small soma area of apical neurons is regulated by NT3.

Supporting cells role in the survival of Spiral Ganglion Neurons

In this present study, the cochlear hair cells and their surrounding supporting cells profoundly affect the morphological phenotype of spiral ganglion neurons. Therefore, it is important to understand the role the surrounding supporting cells play in mediating different aspects of spiral ganglion neurons.

The issue is whether spiral ganglion survival depends on the trophic support provided by the hair cells, the supporting cells or a combination of the two elements. Different observations from animal models with hearing loss due to acoustic trauma or ototoxic drugs suggests spiral ganglion degeneration correlate to the location of inner hair cell loss (Bohne and Harding, 2000; McFadden et al., 2004; Leake and Hradek, 1988; Zimmermann et al., 1995). However, the time progression and the degree of neuronal loss are variable even with the complete loss of the hair cells. Studies report the supporting cells are important to neuronal survival. Sugawara and colleagues examine the role of supporting cells in the long term survival of spiral ganglion neurons after IHC loss in chinchillas from carboplatin (chemotherapeutic agent) treatment or aminoglycoside treatment in cats. The treatment with carboplatin the degeneration is restricted to the IHC. This data shows the survival of a small population of neurons in a region where the

supporting cells are still present. A small population of IHCs is shown to support the survival of a larger amount of neurons for a prolonged period of time. This suggests the IHCs can support the survival of neighboring neurons it does not contact or interactions between IHCs and supporting cells induce the release of a trophic factor to support spiral ganglion survival. Further analysis indicating the role of supporting cells in neuronal survival is from reports using aminoglycoside to induce loss of IHC and different levels of supporting cell loss. Aminoglycoside treatment usually destroys both the IHCs and the supporting cells. The result revealed that regions of supporting cell loss also correlate with neuronal cell loss (Leake and Hradek, 1988; Sugawara et al., 2005).

The drawback of using ototoxic drugs or acoustic trauma to examine the hair cells and their supporting cells role in the regulating the survival of spiral ganglion neurons. Kujawa and Liberman report that acoustic trauma can lead to killing the spiral ganglion neurons even when the hair cells are still intact (Kujawa and Liberman, 2009). The use of ototoxic drugs is reported to damage nerve terminals, which show signs of degeneration even before the IHCs (Wang et al., 2003; Hirose et al., 2004). However, a study evaluates IHC loss on neuronal survival without the damaging effects of noise or ototoxic drugs. The mice lacked the gene for the high affinity thiamine transporter as a result when feed a thiamine rich diets their cochlear structure is normal whereas, a low thiamine rich diet causes a loss of IHCs (Zilberstein et al., 2012). They report spiral ganglion neurons survive and maintain their peripheral projections three months after IHC loss. This indicates that the supporting cells play a role in the long term maintenance of spiral ganglion neurons. The inner border and inner phalangeal cells that surround the IHC is important to the survival of spiral ganglion neurons (Stankovic et al., 2004).

The signaling pathway involved in supporting cell and neuronal interactions is the neuregulin-erbB signaling pathway (Watanabe et al., 2010; Chen et al., 2003). The spiral ganglion neurons express neuregulin (NRG; Morley, 1988;Hansen et al., 2001;Stankovic et al., 2004) and the supporting cells, hair cells, and the Schwann cells express erbB receptors (Stankovic et al., 2004; Hansen et al., 2001; Morley, 1988; Hume et al., 2003). The neuregulins are the growth factors that activate the tyrosine kinase receptors erbB2, erbB3, and erbB4 which are members of the epidermal growth factor (EGF) family. The erbB2 receptor does not have a ligand-binding site

(Klapper et al., 1999). The neuregulins bind to the erbB3 or erbB4 receptors that form heterodimers with the erbB2 receptor (Macdonald-Obermann et al., 2013). The supporting cells within the organ of Corti immunostained for erbB2 and erbB3 (Stankovic et al., 2004). The labeling was most apparent in the inner border cells and phalangeal cells around the IHCs (Stankovic et al., 2004). In addition to the supporting cells beneath the OHCs, which includes the deiters cells, pillars cells, the Boettcher cells, and the inner sulcus cells (Stankovic et al., 2004).

Transgenic mice are used to investigate the role of NRG1-erbB signaling in the mature cochlea. They express the dominant-negative erbB4 receptor in supporting cells under the control of the GFAP (glial fibrillary acidic protein) promoter. The dominant negative-erbB4 blocks the ligand from activating erbB2 and erbB3 receptors. The supporting cells and the organ of Corti remain unchanged this suggests that erbB signaling is important to the function of supporting cells and not survival. The neuronal loss is rapid and restricted to only type I spiral ganglion neurons. This indicates a molecular change in the supporting cells can directly influence neuronal survival which supports the view of the supporting cells are important in spiral ganglion neuron survival in the mature cochlea.

The neurotrophic factor NT3 expression is reduced in the transgenic mice cochlea (Stankovic et al., 2004). However, in the transgenic cochlea it is not certain whether NT3 is specifically expressed in the supporting cells, or the dominant negative-erbB4 expression in the supporting cells may result in the reduced expression of NT3 in the hair cells. Sugawara and colleagues have reported NT3 expression in the supporting cells (Sugawara et al., 2007). This NRG-erbB model suggests the erbB receptors in the supporting cells are activated by NRG1 produced by the spiral ganglion neurons. This results in the release of NT3 which promotes the survival of the type I spiral ganglion neurons. In all, this is a critical debate in the auditory field and the results previously described the supporting cells are important to the survival of spiral ganglion neurons. The non neuronal cells surrounding the organ of Corti provides trophic support to the spiral ganglion neurons but other non sensory cells also play a role in regulating other phenotypic properties.

Mesenchyme Role in Axon Bundling

Spiral ganglion neurons interact with multiple populations of non-neuronal cells. Before synapsing with the hair cells, the peripheral axons of spiral ganglion neurons are guided to their sensory receptors by a variety of non sensory cells types. The developing spiral ganglion neurons extend its peripheral axon through auditory glia and a combination of non-sensory cell types referred to as the otic mesenchyme.

Studies examining the mesenchyme primarily focused on inner ear formation such as capsule development (Liu et al., 2002, 2003; Phippard et al., 1998; Braunstein et al., 2008). In vitro studies, report the periotic mesenchyme is a requirement for sensory hair cell differentiation utilizing dissociated cultures (Doetzlhofer et al., 2004). Montcouquiol and Kelly confirm this result using intact cochlear epithelium (Montcouquiol and Kelly, 2003). Not much is known about the mesenchymal cells involvement in axon guidance however, Coate and colleagues has examined the otic mesenchyme in regulating radial bundle formation. The peripheral axons of spiral ganglion neurons extend through the otic mesenchyme and form a series of radial bundles. Coate and colleagues identified the factors that regulate radial bundle formation. The otic mesenchymal cells play a role in mediating the peripheral innervation bundle pattern within the cochlea (Coate et al., 2012).

The POU domain (Pit1-Oct1/2-unc86) proteins are a family of transcriptions factors that are expressed during inner ear development (Samadi et al., 2005;Phippard et al., 1999;Xiang et al., 1997). The POU domain is a bipartite DNA-binding domain that consists of two conserved regions the POU-specific domain and the POU homodomain. These transcription factors regulate multiple developmental processes such as early embryogenesis, lineage progression, terminal differentiation, and the development of the nervous system (Ryan et al., 1991; Liu et al., 2000; Mu et al., 2004). In the ear, studies examining Pou3f4 report the expression is limited to the otic mesenchymal cells and not in the hair cells or the spiral ganglion neurons (Minowa et al., 1999; Phippard et al., 1998; Samadi et al., 2005).

The Pou3f4 transcription factor expression in the otic mesenchyme is required to promote

the formation of the inner radial bundles. Coate and colleagues used microarray transcriptome screening and mutant analysis to identify that Pou3f4 controls the expression of EphA4 in the otic mesenchyme. The ephrin proteins and their Eph receptor tyrosine kinases play an important role in axon pathfinding (Pickles et al., 2002; Kullander et al., 2001). The Eph receptors bind to cell membrane attached ligands called ephrins. The Eph receptors are divided into A class receptors that bind to A class ligands (GPI –linked to the cell membrane) whereas, B class receptors bind to B class ligands (transmembrane proteins) (Kullander et al., 2001). The exception to this rule is ephrin-B2 and ephrin-B3 can activate EphA4 receptor. The EphA4 is expressed in otic mesenchyme cells and their receptors are expressed on the surface of the spiral ganglion neurons. The EphA4 activates ephrin-B2 expressed by the spiral ganglion to promote radial bundle formation (Coate et al., 2012). The mechanism is not known how ephrin-B2 promotes bundling. It has been suggested that ephrin-B2 reverse signaling may induce axon association with adjacent axons by signaling other factors such as integrins.

Integrins are the principal cell surface receptors that bind extracellular matrix proteins including collagen, laminins, and fibronectin. These receptors are involved in the linkage between the extracellular matrix and the cytoskeleton of the cell. Studies have suggested that integrins can function in cell adhesion (Zusman et al., 1993; Gotwals et al., 1994). The fruit fly Drosophilia melanogaster and the Caenorhabditis elegans have less integrin genes than vertebrates. These two models systems are used because the genetic analysis is simpler. Zusman and colleagues show Drosophilia position-specific (PS) integrins are important for the link between the fenestrated membrane and the basement membrane of the retina (Zusman et al., 1993). Also Gotwals and colleagues show mutated Drosophilia (PS) integrins have cell adhesion defects (Gotwals et al., 1994). These results are consistent to the known role that intergrins play in the cell. The role of integrins has not been previously described to be involved in axon fasciculation. Baum and Garriga demonstrate the first genetic evidence that C. eleglans inα-1 a integrin may play a role in axon bundling. The axons in the inα-1 mutant grew to appropriate lengths but the bundling of the axon showed defects in two separate locations. The anti-GABA stain demonstrated defasciculation of the axons in the ventral nerve cord and in the amphid neurons in

the head (Baum and Garriga, 1997). This result provides another possible role for integrins in mediating axon bundling.

A possible mechanism for intergrins to mediate cell adhesion in an Ephrin protein/Ephrin receptor system. Davy and Robbins utilizing an ectopic expressing system demonstrated ephrin-A5 binding to the Eph receptor induces the activation of β1 integrin, which leads to an increase in cell adhesion (Davy and Robbins, 2000). The ephrin-A may regulate integrin function by the inside-out signaling pathway involving members of the Src family of protein tyrosine kinases. The inside out signaling pathway is the conformational change of the extracellular domain of the integrin receptor when bound to its ligand. The inactive state of the integrin have a low affinity for their ligands but when it is activated their affinity for the ligand increases (Shen et al., 2012). When ephrin-A5 activates β1 integrin it can modulate the adhesive properties of this receptor (Davy and Robbins, 2000). In all, the mesenchyme is important to the regulation of the distinct bundling patterns observed in spiral ganglion neurons.

In this present study, we ask the question of what regulates the distinct axon outgrowth profiles observed in the synapse cultures. We provide evidence from the experiments herein, of non-neuronal cells within the cochlea and the hair cell micro-isolates may play a role. We do not know the identity of these non-neuronal cells but it is present within the region the axons bundle.

The Regulation of the different Tonotopic properties of Spiral Ganglion Neurons

To understand the initial stages of signal coding carried out by the spiral ganglion neurons. The Davis' Lab has examined this issue through different studies that characterized their firing features, threshold levels, and presynaptic and postsynaptic protein levels. Initially, Mo and Davis used electrophysiological studies to examine the heterogeneity in the firing features and ionic currents in spiral ganglion neurons (Mo and Davis, 1997). The neurons are examined with whole cell current clamp that reveal two classes of neurons those with rapid adaptation and those with slow adaptation to prolonged depolarization. The next question explored whether the heterogeneity of spiral ganglion neurons are associated with the tonotopic map in the cochlea. Adamson and colleagues demonstrate that neurons isolated from different cochlear regions had

different electrophysiological properties. Neurons isolated from the apex had longer action potential durations, prolonged latencies to firing, and slower accommodation rates. While, neurons from the basal region had brief action potential durations, abbreviated latencies, and faster accommodation rates. They show the voltage-gated ion channels that control the firing characteristics vary tonotopically. Immunocytochemical staining is completed on postnatal spiral ganglion neurons in vitro and paraffin-embedded tissue sections from adult cochleae. This analysis revealed clear differences in ion channel distribution between apical and basal neurons. The four different voltage-gated K^+ ion channel α-subunits have a role in regulating accommodation, latency, and duration are as follows: the large conductance Ca^{2+}-activated K^+ channel ($K_{(Ca)}$), the low threshold delayed rectifier $K_v1.1$ subunit, the high threshold delayed rectifier $K_v3.1$ subunit, and the A-type (rapid inactivating) $K_v4.2$ subunit. The first three ion channels that contribute to rapid accommodation and reduced action potential duration are mainly located in the basal neurons. The last ion channel, $K_V4.2$ contribute to prolonged latencies are primarily in apical neurons (Adamson et al., 2002a).

The next question addressed whether the electrophysiological features are regulated by extrinsic factors. The response of neurotrophins is evaluated on the neuronal firing patterns and the ion channel distribution. The result revealed BDNF and NT3 have an opposite response on these different characteristics (Adamson et al., 2002b). For example, neurons isolated from either frequency region after the application of BDNF resemble a characteristic basal neuron. Moreover, neurons isolated from either frequency region after the application of NT3 resemble a characteristic apical neuron (Adamson et al., 2002b). This result suggests that in the postnatal cochlea opposite gradients of neurotrophins play a role in regulating the electrophysiological properties of the spiral ganglion neurons. Additional studies explored neurotrophins response on other characteristics such as the distribution patterns of pre- and post- synaptic proteins. The results revealed presynaptic proteins, synaptophysin and SNAP-25, mainly in the apical region are up regulated by NT3 and down regulated by BDNF (Flores-Otero et al., 2007). The postsynaptic proteins, AMPA receptor (AMPAR) α-subunits (GluR2a and GluR3), predominantly in the basal region, is up regulated by BDNF and down regulated by NT3 (Flores-Otero et al.,

2007). These additional tonotopic features are regulated by BDNF and NT3 in an opposing manner (Flores-Otero et al., 2007). Not only do neurotrophins influence the pre- and -post synaptic protein distribution along the tonotopic gradient, but they also regulate the neuronal firing patterns and ion channel composition that mediate action potential transmission. The tonotopic features (regulated by BDNF and NT3) reveal that different intracellular signaling mechanisms mediate different responses through differentially activated Trk receptors.

The tonotopic relationship of the firing characteristics and synaptic protein distribution is reinforced utilizing the hair cell micro-isolate co-culture preparations. The paired spiral ganglion neurons adopted the characteristic feature of the frequency specific region of its hair cell micro-isolate. This unequivocally demonstrates that the peripheral target tissue regulates the phenotypic changes in spiral ganglion neurons. To identify the factors that regulate the actions of the hair cell micro-isolates, we utilized function-blocking antibodies. In the apical region, NT3 is responsible for the enrichment of presynaptic proteins due to the anti-NT3 function blocking antibody inhibited the influence of the apical hair cell micro-isolates. In the basal region, BDNF is responsible for the enrichment of postsynaptic proteins due to the anti-BDNF function blocking antibody inhibited the influence of the basal hair cell micro-isolates. This result shows BDNF regulates features predominantly in the base whereas; NT3 regulates features in the apex.

i. The Regulation of Soma Area and Axon Outgrowth in Spiral Ganglion Neurons

From the studies previously described the electrophysiological properties and synaptic protein levels are graded systematically along the cochlear frequency map. In the base of the cochlea, the protein levels of the glutamate receptors (GluR2a and GluR3) are enhanced indicating these receptors could mediate high frequency transmission. In the apex of the cochlea, the protein level of synaptophysin is enriched indicating this factor could mediate low frequency transmission. Moreover, the ion channel composition that regulates the differential firing properties of the apical and basal neurons is graded along the tonotopic axis. These graded specializations along the frequency axis are regulated by the neurotrophins BDNF and NT3. Therefore, features predominant in the apical region are upregulated by NT3, whereas, features

mainly in the basal region are upregulated by BDNF. We investigated whether the morphological properties of spiral ganglion neurons is regulated by this same regulatory mechanism. This present study is specifically focused on evaluating the different tonotopically-varying morphological features of postnatal spiral ganglion neurons such as soma area and axon length. Despite the importance of these morphological features in regulating signal transmission speed, the factors involved in its regulation are not known.

The peripheral target tissue has a profound impact on regulating the soma area of spiral ganglion neurons. For example, the large cell body of the basal neurons is significantly reduced when paired with hair cell micro-isolates excised from the apical region of the cochlea. Under the same culture conditions, an anti-NT3 function blocking antibody is used to determine whether NT3 is responsible for the reduction in soma area. This result reveals that NT3 is a significant regulator of cell size. With the small cell body of apical neurons, the opposite is observed; it significantly increases when paired with hair cell micro-isolates excised from the basal region of the cochlea. Under the same culture conditions, an anti-BDNF function blocking antibody is used to determine whether BDNF is responsible for the enhancement of soma area. This result shows that BDNF is a significant regulator of spiral ganglion neuronal phenotype. The neurotrophic factors BDNF and NT3 are significant regulators of spiral ganglion soma size. However, when isolated apical and basal neuronal explants are directly exposed to BDNF and NT3; the relative size of the neurons remained unchanged. When comparing the, neuronal explant to the co-culture preparation, it is plausible that other factors secreted by the hair cells (or their surrounding satellite cells) contribute to regulating soma area.

In addition to significantly altering soma area, the hair cells dramatically impact axon ratio length profiles as well. In the co-culture preparations, the axons demonstrate putative PNS and CNS axon characteristics. For this reason, the analysis of the PNS/CNS axonal length ratios is conducted. The result demonstrates the effect of the hair cell micro-isolates on the axon ratio length is modulated by BDNF and NT3.

Altogether, the neurotrophic factors BDNF and NT3 differentially regulate soma area and axon outgrowth. These findings indicate the structural features of spiral ganglion neurons have a

different regulatory mechanism in comparison to the synaptic protein and the voltage-gated ion channel composition.

METHODS

Tissue cultures. All cell cultures were maintained in growth medium: DMEM (Sigma D6171) with 10% fetal bovine serum, 4mM L-gulatamine, and 0.1% penicillin-streptomycin. Neurons were maintained in culture at 37ºC in a humidified incubator with 5% CO_2. Procedures performed on CBA/CaJ mice were approved by The Rutgers University Institutional Review Board for the Use and Care of Animals (HRB-UCA), protocol 90-073. Postnatal day 5-9 mice were euthanized and decapitated to obtain the inner ear tissue. The inner ear tissue was used to prepare different culture preparations: paraffin-embedded sections, organ of Corti and innervating spiral ganglion neurons, neuronal explant and the gangliotopic culture.

Neuronal explant culture – In the first preparation one-fifth of apical and basal spiral ganglion neurons taken from CBA/CaJ mice was placed in culture dishes as intact explants and maintained for 6-7 days *in vitro* (DIV) (Adamson et al., 2002). We analyzed the effect of neurotrophins on soma area by supplementing explant tissue with NT3 (PeproTech, 450-03), BDNF (PeproTech, 450-02) or combinations of BDNF and NT3. The neurotrophins was added at the initial isolation and plating of the tissue culture. The explant cultures were approximately the same size. The following NT3 concentrations were added to the explant cultures: 0.01ng/ml, 0.1ng/ml, 0.25ng/ml, 0.5ng/ml, 1ng/ml, 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml. The vehicle was 10ul of water or media the amount used for the 10ng/ml of neurotrophin. BDNF used at the following concentrations: 1ng/ml, 5ng/ml, 10ng/ml, 50ng/ml, and 100ng/ml. The BDNF vehicle was 10ul of water or media the amount used for the 10ng/ml of neurotrophin. The ratios of BDNF and NT3 used at the following concentrations: 2.5ng/ml BDNF /7.5ng/ml NT3, 7.5ng/ml BDNF / 2.5ng/ml NT3, 5ng/ml BDNF / 5ng/ml NT3, and 10ng/ml BDNF/ 10ng/ml NT3. The ratio of BDNF and NT3 the vehicle was 400ul of media the amount used for the 10ng/ml BDNF / 10ng/ml NT3. The control in the neurotrophin concentration series experiments was averaged between the vehicle and the untreated conditions. This approach was taken because the soma area was similar in size.

Explant tissue cultured for 10div to examine axon outgrowth patterns. The orientation of the

explant was labeled to distinguish peripheral and central regions. Explant cultures were supplemented with 5ng/ml BDNF or 5ng/ml NT3 to enhance axon outgrowth patterns.

Gangliotopic culture – The second preparation is the gangliotopic culture (GN), in which the entire spiral ganglion was placed in a tissue culture dish. The relative positions of the neurons were maintained along the length of the cochlea. This preparation is in culture for 4 DIV.

Synapse culture – An explant of spiral ganglion neurons paired with regions of the organ of Corti along with their surrounding satellite cells (micro-isolates) this preparation is called a synapse culture. This culture consists of a strip of inner and outer hair cells along with their micro-isolates placed near a spiral ganglion neuronal explant. The hair cells were removed as an intact strip, therefore the alignment of the single row of inner hair cells and three rows of outer hair cells were maintained. By separating the tissue in this way we are able to mix- and -match spiral ganglion neuronal explants with hair cell micro-isolates taken from different frequency regions. The preparations were used from 10-18 div. This time is needed for the processes to grow and reform connections with the organ of Corti micro-isolates. Cultures were analyzed without neurotrophins addition, or BDNF and NT3 together or separately, each at 5ng/ml, to evaluate neuronal morphology. Select cultures were supplemented with function blocking antibodies (anti-NT3 function blocking antibody (R&D Systems, AF-267-NA, 2ug/ml), anti-BDNF function blocking antibody (Promega, G1641, 4ug/ml), anti-NGF receptor p75 antibody (Millipore, 1ul/ml) added at the time of isolation and at 4 day intervals.

In a subset of synapse culture experiments two mouse strains were used because each had a specific attribute that was needed to track the fibers. The mice were two Thy1-YFP mouse strains (B6.Cg-Tg(Thy1-YFP)16Jrs/J, Jackson Labs and Thy1-YFP12Jrs/J which was generously provided by Dr. Jianxin Bao from Washington University. Each YFP mouse strain had a different beneficial attribute. The Thy1-YFP12 mice expressed YFP in all the spiral ganglion neurons. The drawback was the expression was finite. The Thy1-YFP16 mice expressed YFP in only a few neurons and their processes. The small number of neurons that expressed YFP enabled the

tracking and analysis of individual processes.

Organ of Corti culture – The fourth preparation is the organ of Corti (OC) this includes the spiral ganglion neurons with its peripheral innervation still intact. This preparation was cultured for 2 or 3 DIV.

Organ of Corti - Cochlear Nucleus culture–In this new preparation, the OC was cut into three separate regions apex, middle and base with its connection to the brain still intact.

Paraffin-embedded sections- Postnatal animals at the age 5-7 from CBA/CaJ mice. The temporal bones were removed and placed in 10% formalin or 100% methanol for 45 minutes. The tissue was washed three times with 0.01M PBS (pH 7.4) for 15 minutes each or overnight at room temperature. The postnatal temporal bones were dehydrated in 50%, 75%, 80%, and 95% ethanol for 1 hour each. This was followed by 95% ethanol for another 1 hour, 100% ethanol for 30 minutes, and n-butanol for overnight at room temperature. The next day the tissue was placed in fresh n-butanol for another 2 hours. Lastly, the temporal bones were embedded in paraffin and sectioned at 4-6mm in thickness. The tissue was placed on a poly-L-lysine-coated glass slides (VWR; 48311-703), dried overnight and stored at room temperature.

Immunofluorescence. The tissues cultures were fixed with 100% methanol, washed and incubated at room temperature (RT) for 1 hour in 5% normal goat serum (NGS) or 5% nonfat dry milk. The primary antibodies were than applied and incubated for 24 h at 4°C. The specificity of the primary antibodies used in this study was characterized and listed in the JCN Antibody Database: polyclonal and monoclonal class III β-tubulin, polyclonal and monoclonal calbindin, HCN1, HCN4, MAP2, $p75^{NTR}$ antibodies to extracellular and intracellular domains, and synaptophysin. The specificity of anti-Sortilin antibody was examined in liver tissue with siRNAs targeted to the SORT1 gene (Musunuru et al., 2010). The primary antibodies were summarized in Table 1. After washing with 0.01 M PBS, pH 7.4, the tissue cultures were incubated for 1 hour at

room temperature with a fluorescent-conjugated secondary antibody at 1:100. The secondary antibodies from Invitrogen were the following: Alexa-Fluor 488-conjugated anti-rabbit secondary antibody (A-11070), Alexa-Fluor 594-conjugated anti-mouse secondary antibody (A-11020), Alexa-Flour 488 conjugated anti-mouse secondary antibody (A-11017), Alexa-Flour 594-conjugated anti-rabbit secondary antibody (A-11072), Alexa-Flour 350-conjugated anti-rabbit secondary antibody (A-11069), Alexa-Flour 350-conjugated anti-mouse secondary antibody (A-11068), and Alex Fluor 488-donkey conjugated anti-goat secondary (A-11055). The tissue preparations were then washed and mounted in DABCO.

Imaging. The Hamamatsu 1394 Orca-ER acquired all the images utilizing the IPLab Scientific Imaging Software (BD Biosciences). Exposures times were maintained throughout the imaging process. The IPLab Scientific Imaging Software was used complete the analysis for soma and nuclear area, survival and axon bundle length.

Quantitative analysis

Soma and nuclear area analysis. Soma and nuclear area measurements were accurately reflected with anti-β-tubulin. The soma area measurements from anti-β-tubulin were essentially identical to the extracellular label of anti-hyperpolarization activated cation α-subunit 1 (HCN1) antibody. This allowed measurements to be made from anti-β-tubulin, which had the advantage of labeling all cells essentially uniformly, while most other antibody labeling, including anti-HCN1, was relatively heterogeneous. The nuclear area was labeled with either 4'6'-Diamidino-2-phenylindole dihydrochloride (DAPI; D9542, Sigma-Aldrich, St. Louis, MO) or Hoechst dye (33342,Sigma-Aldrich, St. Louis, MO) which showed a similar nuclear area when compared to the unlabeled area from the anti-β-tubulin antibody. This analysis showed that anti-β-tubulin could be used to accurately measure both the soma area membrane and nuclear size.

[Figure 1]

Each image was normalized separately to obtain the best picture for measurements. Soma area

and nuclear measurements consisted of outlining each individual neuron by hand in IPLab. The soma and nuclear area must be clearly visible for an accurate measurement.

Survival analysis. The neuronal survival measurements for apex and base explant cultures consisted of counting all the neurons in the culture dish.

Peripheral and Central Ratio analysis. In the synapse culture, the axon bundle length was measured from the center of the cluster of spiral ganglion neurons to the end of the axonal bundle.

Axon bifurcation analyses in explant cultures. The neurites in the neuronal explant culture was measured using Image Processing and Analysis in Java Software (ImageJ). The measurement was taken from the center of the cell body to the first bifurcation point. Each image was normalized separately to obtain the best picture for measurements. The statistical comparisons were made using a Student's two-tailed paired *t*-test. SEM is indicated in the figures by error bars and in the text after the (±) symbol.

RESULTS

This study characterized the hair cells role in regulating the different morphological properties of spiral ganglion neurons. We examined the soma area and the PNS/CNS axon outgrowth characteristics that varied with tonotopic position; therefore we questioned whether these different structural features were regulated by neurotrophins, which are also graded along the contour of the cochlea. Initially established soma area and axon outgrowth in postnatal animals; than identified whether neurotrophins were involved in their regulation by utilizing different culture preparations.

Chapter I: Soma Area

The classically bipolar and pseudomonopolar cell body shapes of spiral ganglion neurons, also observed in *vitro,* were all included in the soma area analysis (Fig. 2A). A small sub-set of neurons referred to as Type III spiral ganglion neurons (Romand and Romand, 1987), were also observed in apical regions were identified by their large ratio of cytoplasm to nuclear area than was typical for most other ganglion cells (Fig. 2B). The large type III neurons were excluded from the averaged data set because they were above 3 standard deviations above the mean. An initial examination of postnatal neurons isolated from either the apex or base of the ganglion showed a range of cell body sizes within each region, however, there were clear overall size differences between these neurons *in vitro* (Fig. 2C-H). We constructed frequency histograms of apical and basal neurons from a single experiment. In the plots, neurons isolated from the extreme apical and basal regions demonstrated a wide range of cell body sizes (Fig 2I-J).

[Figure 2]

While the soma area measurements were confined to the enlarged somatic region, it was recognized that the specialized somata area extended beyond this area. This specialized region, which possessed a myriad of voltage-gated ion channels (Hossain et al., 2005; Fryatt et al., 2009; Gong et al., 1999; Boiko et al., 2001), were demarcated by microtubule-associated protein 2 (MAP2), a known dendritic protein (Teng et al., 2001;Kosik and Finch, 1987). The anti-hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (HCN4; Fig. 3E) was localized in the same region as anti-MAP2 (Fig. 3D) at the initial processes flanking the cell body and anti-β-tubulin showed the extent of the process (Fig. 3F). In addition to HCN4, the voltage-gated calcium channel the (N-type) $Ca_v2.2$ α-subunit (Chen et al., 2011) and anti-Ca^{2+} activated potassium channel (BK) were observed to label this same region within the spiral ganglion neurons. Loane and colleagues, showed the co-expression of the N-type $Ca_v2.2$ and BK channels, suggesting a potential functional relationship (Loane et al., 2007). This similarity in the distribution pattern characterized by the anti-MAP2 antibody indicates a relationship between these ion channels within this specialized region.

Flanking the initial processes labeled by anti-MAP2 were anti-Ankyrin G (AnkG) immunolabeled patches which most likely defined the nodal region that surrounds the cell body (Fig. 3A,B). AnkG, characterized *in vivo* in Purkinje neurons showed that this adaptor protein coordinated the assembly of the proteins localized at the axon initial segment such as voltage gated sodium channels (Jenkins et al., 2001). Measurements of the luminance intensity from both staining patterns of AnkG and MAP2 were analyzed and correlated with distance (μm) the line (Fig. 3C, green, AnkG; red, MAP-2). The analysis from apical and basal spiral ganglion explant cultures showed that these features were, unfortunately, not reliably reproduced *in vitro*. The extent of the MAP2 extension into the processes and the presence or absence of the AnkG patches varied widely from cell-to-cell and from culture-to-culture, most likely depending on multiple factors, in addition to days *in vitro.* Thus, while, the distribution patterns of anti-MAP2 and anti-AnkG antibodies highlight the electrophysiological importance of the cell soma, we did not further characterized these somatic regions further herein.

[Figure 3]

While the tonotopically-related soma area of spiral ganglion neurons has been clearly established in adult animals *in vivo* (Berglund and Ryugo et al., 1991; Nadol et al., 1990;Rosbe et al., 1996), it has not yet been established whether this was also the case earlier in development. Therefore, to determine whether acute postnatal neurons display similar soma area differences. Soma area measurements were made from postnatal neurons in paraffin-embedded sections from multiple preparations examining four different cochlear locations: apical, mid-apical, mid-basal, and basal regions. These measurements showed postnatal neurons have the same tonotopic soma size differences observed in the adult animal. Apical neurons have a smaller soma area when compared to neurons localized in the basal region (Fig. 4A-D).

Next, the soma area was systematically examined across the tonotopic ganglion; the measurements were completed using a gangliotopic culture (see Methods) which was maintained for four days *in vitro*. The size distribution of spiral ganglion neurons across the ganglion showed a similar soma size pattern observed in the postnatal paraffin-embedded sections. The extreme basal neurons possessed the largest soma area. The neurons were smaller in the mid-basal,

middle, regions and further decreased towards the apical region (Fig. 4E-H). This result revealed the complex distribution pattern of soma area along the cochlea contour. The heterogeneity of the cell size, shown above for the explant cultures (Fig. 2), was also noted in each frequency region. Moreover, measurements of soma area from the extreme apical and basal regions in neuron explants, cultured for 6 div, also retained the soma, as well as nuclear area differences (Fig. 4I-K).

[Figure 4,2]

While the relative soma area differences between regions in the different preparations were consistent, there was a difference in the absolute soma size values that varied greatly between the paraffin-embedded sections, neuron explants, and gangliotopic preparations. We expected that these differences were related to multiple factors, including the increase in soma size that has been observed over time *in vitro* (Zhou et al., 2005) and the tissue shrinkage that occurs during the embedding process to prepare cochlear sections (Ross, 1952; Boonstra et al., 1983). Despite the absolute size differences noted between different preparations, the basic observation of tonotopically-associated soma size of postnatal neurons was consistent from preparation to preparation and with observations from adult sections.

The role of BDNF and NT3 in the regulation of soma area

Because the postnatal culture preparations retained the basic soma area relationship to tonotopy, we utilized these preparations to examine the role of neurotrophins on soma area when isolated from their peripheral and central targets. Initially, measurements of soma area were made at the specific neurotrophin concentration that unequivocally altered the electrophysiological firing properties and density of the voltage-gated ion channels (Kv4.2, Kv1.1, $K_{(Ca)}$ and Kv3.1; Adamson et al., 2002). The synaptic protein distribution of postsynaptic AMPA receptors (GluA2,3) and presynaptic proteins (synaptophysin and snap-25) (Flores-Otero et al., 2007) were altered at this concentration. Measurements at 5ng/ml, however, showed no change in the soma area of apical and basal neurons compared to control conditions (Fig. 5A), while the relative size differences between apical and basal neurons were still retained.

In order, to compare the soma area analysis completed in the highly controlled culture conditions to those reported in the intracochlear infusions of BDNF into the deafened cochlea (without the full complement of the hair cell receptors) where basal neurons size increase were noted (Leake et al., 2011, McGuinness and Shepherd, 2005; Shepherd et al., 2005; Agterberg et al., 2008). To identify whether BDNF could increase cell size as suggested by the intracochlear infusion experiments, we next evaluated the role of BDNF at a wide range of concentrations, including those higher than standard physiological levels (1, 5, 10, 50, 100ng/ml; Fig. 5A). By increasing the concentration by 20-fold, we observed much to our surprise that other than a slight enhancement at 10ng/ml, which was not significant, the absolute soma size of basal neurons were maintained. The relative size differences between apical and basal neurons were also essentially maintained at all concentrations tested. However, at 10ng/ml of BDNF a significant decrease in apical soma size ($193\mu m^2$ ± 3.7, n=10; $p<0.05$; Fig. 5A) was noted when compared to the control. The soma area differences between apical and basal neurons showed a significant difference at the 10ng/ml of BDNF no other concentrations analyzed reached significance (Fig. 5D). While, BDNF evaluated at the different concentrations had only a minor effect on soma area, it had a significant impact on neuronal survival.

NT3 was shown to have a greater impact on altering cell size at a wider range of concentrations than BDNF. The concentration that showed in previous studies to significantly affect the firing patterns and the synaptic protein distribution in spiral ganglion neurons (Flores-Otero et al., 2007; Adamson et al., 2002), 5ng/ml of NT3 significantly altered soma area, but in apical neurons alone. Similar to that shown in retinal ganglion cells (Lom and Cohen-Cory, 1999) NT3 actually *reduced* the soma area in apical neurons (0.1, 0.25, 0.5, 1, 5, 10, 50, 100ng/ml). The basal neurons remained unchanged or showed a slight, but non-significant increase in size at the higher NT3 concentrations (Fig. 5B). As a result, the difference in soma area between apical and basal neurons was enhanced at the higher concentrations (Fig.5E).

[Figure 5]

Survival measurements of BDNF at the same concentrations utilized for soma area showed an increase in apical and basal neurons. Apical neurons at a concentration as low as 1ng/ml enhanced neuronal survival above control ($p<0.01$); while the maximum survival response was achieved at concentrations of 10 to 50ng/ml (Fig. 6A). Also, BDNF significantly enhanced the survival of basal neurons at 5, 10, 50 and 100ng/ml compared to the control (Fig. 6A). The effect of BDNF on the survival of apical and basal neurons was very robust, which is in contrast to the limited impact BDNF had on soma area. The difference in survival between apical and basal neurons was significant at all concentrations (Fig. 6D). Interestingly, at any concentration analyzed apical neurons consistently showed a greater enhancement in survival over basal neurons.

The survival measurements in cultures supplemented with NT3 showed an enhancement at the same concentrations that significantly reduced apical neuronal area (Fig. 6B). Apical neurons survival was significantly enhanced at a concentration as low as 0.1ng/ml when compared to control ($p<0.01$;Fig. 6B); it plateaued at concentrations of 5ng/ml to 10ng/ml (Fig.6B). While, in basal neurons the 0.25ng/ml concentration of NT3 significantly enhanced survival when compared to control ($p<0.01$;Fig. 6B). NT3 was able to increase survival at very low concentrations for apical and basal neurons. The difference in survival between apical and basal neurons showed an increase with concentration until it peaked at 5ng/ml and dropped at the higher concentrations (Fig. 6E). In fact, both NT3 and BDNF showed increased apical neuronal survival over basal neurons *in vitro*.

[Figure 6]

To determine whether neurotrophins together exert a different effect than when utilized separately, apical and basal neurons were supplemented with different combinations of BDNF and NT3 concentrations. The following concentrations were utilized: in which either NT3 was higher at (7.5ng/ml NT3/ 2.5ng/ml BDNF), BDNF was higher at (7.5ng/ml BDNF/ 2.5ng/ml NT3), or BDNF and NT3 were at the same concentration (5ng/ml BDNF / 5ng/ml NT3) and (10ng/ml BDNF / 10ng/ml NT3). The ratio combinations of BDNF and NT3 didn't change the cell size differences between apical and basal neurons (Fig. 5C). The combination of 5ng/ml of both

BDNF and NT3 showed a slight increase in the base (296.2μm^2 ± 6.6, n=5; Fig.5C) and a slight decrease in apex (199.9μm^2 ± 5.9, n=5; Fig.5C) although these changes were not significant compared to the control. The significant difference was noted between apical and basal neurons at this concentration (p<0.05; Fig. 5F).

The ratio combinations of BDNF and NT3 significantly influenced the survival of apical and basal spiral ganglion neurons compared to control (p<0.01; Fig. 6C). The survival measurements from the ratio combinations showed the same trend of preferential enhancement of apical neurons over basal neurons at each concentration. The difference between apical and basal survival was significant at all concentration tested (Fig. 5F). It was noted that the biggest difference in survival occurred in the ratio combination with the higher NT3 concentration (2.5ng/ml BDNF / 7.5ng/ml NT3).

[Figure 5,6]

These results showed that BDNF and NT3 have distinct effects on the soma area of spiral ganglion neurons *in vitro*. BDNF was shown to reduce the size of apical neurons and slightly increased the size of basal neurons at the 10ng/ml concentration but no other concentrations had any effect. NT3 was shown to only reduce the size of apical neurons at a wide range of concentrations but basal neurons remained unchanged. It was noted that the exogenous treatment of BDNF, NT3, or a combination of these neurotrophins didn't change the cell size differences between apex and base neurons.

Both neurotrophins were shown to only alter the specific size of apical neurons making it smaller and the survival measurements showed a consistent and preferential enhancement of apical neurons. This indicates that the soma area of apical neurons seem to be more sensitive to the effects of both neurotrophins. This reduction observed in the soma area of apical neurons was not due to the cells being unhealthy because survival was significantly increased. Comparing this result to the electrophysiological and synaptic protein composition findings indicates that cell size has a different regulatory mechanism than previous findings because each neurotrophin, either separately or in combination, only changes cell size within a specific frequency region.

The Soma Area of Spiral Ganglion Neurons was altered when paired with distinct regions of the organ of Corti

We next wanted to examine the peripheral targets of spiral ganglion neurons that secrete trophic factors role in the regulation of cell size. Utilizing synapse cultures (see Methods), which were co-cultures of organ of Corti micro-isolates taken from different tonotopic regions and paired with spiral ganglion neuron explants. A low magnification image revealed spiral ganglion neurons (SGNs; labeled with anti-β-tubulin, red and anti-synaptophysin, green) re-innervating the hair cells (HC; labeled with anti-calbindin, blue) and re-formed their synaptic connections; in most cultures, however, only a limited number of peripheral processes do extend into the organ of Corti micro-isolates (Fig. 7A). A closer examination, of the hair cell region, revealed viable synapses were formed (unpublished data). We noted that some of the afferent processes do so in specializations that contain the presynaptic protein synaptophysin. This result suggested that the reciprocal synapses described in adult sections (Thiers et al., 2008; Sobkowicz et al., 2003) were also reproduced *in vitro* (Fig. 7B).

[Figure 7]

Previous studies showed that the organ of Corti micro-isolates taken from either tonotopic region can change the phenotype of synaptic protein distribution (Flores-Otero et al., 2007) in spiral ganglion neurons we wondered whether this would also have an impact on neuronal morphology. The initial step was to assess soma area in preparations in which the micro-isolates and neuron explants were isolated from the same frequency region (Fig. 8A-C). The apical organ of Corti micro-isolates paired with apical neuron explants the soma area was smaller (205.0μm^2 ± 5.2; n=33 p<0.01;Fig. 8A,D) compared to the larger soma area (285.0μm^2 ± 10.9, n=29; Fig. 8C,D) of the basal neuron explants paired with the basal organ of Corti micro-isolates. The middle organ of Corti micro-isolates paired with middle neuron explants showed a slightly larger area (213.1μm^2 ± 8.3, n=16; Fig. 8B, D) compared to apical organ of Corti micro-isolates paired with apical neuron explants. This similar gradation in soma area (Fig. 8D) corresponded to the

variation along the tonotopic gradient already observed in the acute postnatal sections.

To examine whether the organ of Corti micro-isolates directly alter soma area, we mixed – and-matched the organ of Corti micro-isolates by isolating the micro-isolates and explants from different tonotopic regions. By taking this approach we were able to reverse the soma areas of apical and basal neurons. Thus, the neuron somata in apical neuron explants paired with basal organ of Corti micro-isolates significantly increased ($300.4\mu m^2$ ± 14.4, n=17; Fig. 8E,H) when compared to control the difference was significant ($p>0.01$). The levels were similar to the basal neuronal soma measured in matched basal synapse cultures (Fig 8H). Conversely, when we paired basal neuron explants with apical organ of Corti micro-isolates the soma size significantly decreased ($201.9\mu m^2$ ± 3.1,n=20; Fig. 8G,H) when compared to control ($p<0.01$). The levels were similar to the apical neuronal soma measured in matched apical synapse cultures. The soma area decreased when basal neuron explants paired to middle organ of Corti micro-isolates ($216.4\mu m^2$ ± 13.4,n=3; Fig. 8F,H) but it was not significantly different when compared to matched middle synapse cultures.

To determine whether the secretion of BDNF from the basal organ of Corti micro-isolates was responsible for the increased soma area of apical neurons, we utilized anti-BDNF function blocking antibody to block the ligand. Under these conditions we observed a significant reduction in soma area ($257.1\mu m^2$ ± 20.3, n=7; $p<0.05$; Fig. 8H) when compared to control ($p<0.05$; Fig. 8H). To determine whether the secretion of NT3 from apical organ of Corti micro-isolates was responsible for the decreased soma area of basal neurons, we utilized anti-NT3 function blocking antibody (Fig. 8H). In this co-culture the soma area of basal neurons increased significantly ($247.1\mu m^2$ ± 7.8,n=8; Fig.8H) when compared to control ($p<0.01$; Fig.8H). These results indicated within the context of the organ of Corti micro-isolates, cell size follows the same regulatory mechanisms observed for both synaptic protein distribution and the electrophysiological firing features.

[Figure 8]

While the synapse culture experiments clearly showed a robust effect on soma area which was affected by the presence of BDNF and NT-3, this clearly differs from explant cultures in which BDNF had limited effects and NT-3 reduced soma area predominately in only apical neurons. Thus, altogether, these results suggest that BDNF and NT3 may work in combination with a number of different cells in this co-culture system, such as trophic factors secreted by the hair cells and/or satellite cells as well as direct synaptic connections. Thus, within this culture there are many different elements that could be working in combination with BDNF and NT3 directly or indirectly to mediate soma area transformations that we only observed within the synapse culture.

To understand the interplay of a cofactor(s) with NT3 and BDNF we utilized this same culture preparation with organ of Corti micro-isolates and explants taken from the same frequency regions with the application of neurotrophins or function blocking antibodies (Fig. 9). Apical neuron explants when paired with apical organ of Corti micro-isolates with the application of NT3 ($201.0\mu m^2 \pm 9.7$, n=8; Fig.9A) or BDNF ($211.9\mu m^2 \pm 7.0$, n=8) showed no significant changes in size compared to control ($205.0\mu m^2 \pm 5.2$, n=33; Fig. 9A). Neither middle neuron explants paired with middle organ of Corti micro-isolates with the application of NT3 ($222.7\mu m^2 \pm 5.6$, n=8;Fig.9A) or BDNF ($205.4\mu m^2 \pm 11.7$,n=8) did not show significant changes in cell size compared to control. However, basal neuron explants when paired with basal organ of Corti micro-isolates with the application of NT3 ($240.5\mu m^2 \pm 8.8$, n=11;Fig.9A) or BDNF ($237.1\mu m^2 \pm 12.6$, n=7) the cell size decreased significantly when compared to control ($p<0.05$). This indicated that only in the basal region a cofactor was required to alter the size of basal spiral ganglion neurons. When compared to the exogenous application of BDNF and NT3 separately or in combination to explant cultures. BDNF could not alone alter the soma size of apical or basal spiral ganglion neurons. This reflected that the trophic factor secreted by the organ of Corti micro-isolates or from the satellite cells. Either directly work in combination with BDNF or indirectly by acting on another cell type that provides the factor needed to change cell size.

[Figure 9]

Survival measurements from matched synapse cultures of apical, middle, and basal organ of Corti micro-isolates paired with spiral ganglion neuron explants are shown in Figure 10. The matched apical organ of Corti co-cultures supplemented with NT3 (102 ± 11.7, n=9; Fig.10A) and BDNF (71 ± 11.6, n=9) was significantly different when compared to control (27 ± 4.0,n=22; $p<0.01$; Fig.10A). Similarly, matched basal organ of Corti co-cultures supplemented with NT3 (101 ± 19.4, n=10) and BDNF (86 ± 31.8, n=3) showed significant differences when compared to control (43 ± 4.7, n=20; $p<0.05$; Fig.10A). While both BDNF and NT3 significantly enhanced survival, NT3 had the largest effect (Fig. 10B), which was consistent with observations reported for neurotrophin survival in neuron explant cultures (Fig. 6B). Furthermore, the blockers had the greater effect within the apical region (Fig. 6A-C) was predicted by the enhanced survival in apical explants**.**

[Figure 10,6]

In conclusion, these findings showed that soma area was clearly regulated differently from the electrophysiological properties and synaptic protein distribution. When BDNF and NT3 were exogenously applied to apical and basal neuronal explants the original sizes were retained, apical neurons maintained a smaller soma area compared to the larger size of the basal neurons. Both neurotrophins were shown to significantly reduce the soma area of apical neurons however, with the enhancement of survival this reduction in soma area was not due to the cells being unhealthy. The synapse culture had an influential effect on soma area that was affected by the presence of BDNF and NT3. Thus, when compared to the neuronal explant cultures supplemented with neurotrophins a cofactor was determined to be involved in regulating soma area. The result showed in the matched apical and basal co-cultures supplemented with either BDNF or NT3 only basal neurons may require a co-factor to alter soma area.

Chapter II: Axon Ratio Differences between the Peripheral and Central region

The axon projection from each spiral ganglion neuron cell body must first navigate through the internal auditory meatus to enter the cochlear nucleus before it bifurcates. Whether axons emanate from a type I or type II spiral ganglion neurons, they extend through the cochlea to a bifurcation point according to the characteristic frequency of the fiber, which is initially determined by its peripheral innervation cite in the organ of Corti (Nayagam et al., 2011; Liberman 1982; Fekete et al., 1984). Thus, like most primary afferents the spiral ganglion is a hybrid neuron having characteristics that span the peripheral and central nervous systems. The peripheral nervous system portion of the fiber that projects from the cell body to the internal auditory meatus is called the intracochlear axon (Liberman and Oliver, 1984). While the central nervous system segment of the fiber from the internal auditory meatus to the bifurcation in the cochlear nucleus is called the root branch (Fekete et al., 1984; Ryugo and Rouiller et al., 1988). In both the peripheral and central nervous systems, two separate cells are involved in myelination; the Schwann cells myelinate the peripheral process and the oligodendrocytes myelinate the central axon. Thus, the length of the intracochlear axon and root branch and its source of myelination are graded according to cochlear location. While the total axon length is essential equal for all neurons in the spiral ganglion, apical neurons have a higher proportion of peripherally-myelinated axon, while the basal neurons have a higher proportion of centrally-myelinated axon.

Variation in the peripheral process indicates the intracochlear axon length

In order, to determine whether organ of Corti micro-isolates regulate other aspects of spiral ganglion neuron morphology in addition to soma area, we examined axon outgrowth in synapse cultures. The mice utilized in all the measured analysis were CBA/CaJ mice, the widely accepted model of normal hearing in the field (Ohlemiller et al., 2010). However, in a sub-group of experiments we utilized two Thy1-YFP mice strains which illuminated spiral ganglion neurons (Fig.11A), thus enabling examinations of live outgrowth to be compared at different times *in vitro* The example shown in Figure 11 compared the explant initially placed in culture (Fig. 11B) to one

observed 12 days later in order to observe the *de novo* outgrowth patterns of the axonal processes (Fig. 11C).

In the synapse culture, the axon projections from both the peripheral and central side of the neuronal somata were excised and placed near the isolated organ of Corti micro-isolates (Fig.11B, arrowheads and dashed line, respectively). After 12 div both excised neuronal processes from the spiral ganglion neurons regrew (Fig.11C, arrowhead and arrows, respectively). The isolated inner and outer hair cell micro-isolates (dashed line) paired with an isolated neuronal explant (arrowheads). The spiral ganglion neurons regenerated both axons; one innervating the hair cells (arrowheads) while the other axon projected away from the explant (Fig.11C). Moreover, there was evidence by the branching patterns within the organ of Corti micro-isolates that Type I and Type II neurons and their processes can retain a similar innervation. The type I pattern, indicated by the double arrowhead, showed local branching within the region of the inner hair cells; the type II pattern, indicated by the single arrowhead, turned and extended within the outer hair cell region of the micro-isolate (Fig. 11C). While these patterns appeared to reiterate the endogenous innervating patterns, we also observed many processes that do not successfully re-innervate the target tissue. Some fibers either turn abruptly (Fig.11D) perhaps due to repulsive signals or miss the micro-isolate entirely by extending long processes well beyond the tissue.

What was most remarkable about the process outgrowth from the spiral ganglion explants, however, was the highly directed axon outgrowth projecting away from the explant ostensive toward its central targets (Fig.11C). These processes extended for 100's of microns beyond the micro-isolates (Fig. 11E) forming either tight or loose bundles (Fig.11C, arrows). The long parent branch eventually bifurcated (Fig.11G, I) and ultimately after further bifurcations terminated into small basic endings (Fig. 11H,J). This culture allowed for the examination of the axonal processes that projected *in vitro* in an orderly fashion away from the organ of Corti micro-isolates. This allowed for the examination of the peripheral target tissue effect on axon length, which may include some of the peripheral processes that sometimes reverses away from the organ of Corti micro-isolate to join the axon bundle group.

[Figure 11]

An example of a typical synapse culture preparation immunolabeled to show the hair cells (anti-calbindin), neurons (anti-β-tubulin) and presynaptic specializations (anti-synaptophysin) was shown in figure 12. This culture preparation showed the axonal processes were highly organized and extended in a uniform direction from the cluster of spiral ganglion neurons (Fig. 12A, SGN). The point at which the processes change direction was referred to herein as the transition zone, which has a high distribution of anti-synaptophysin-labeled puncta (Fig. 12A, C, double arrow heads). The processes extended pass the transition zone change trajectory to travel in multiple directions forming a crisscross pattern where anti-synaptophysin labeled puncta were distributed throughout (Fig. 12A,D, arrow head). The synapse culture reflected observations in-vivo of presynaptic specializations at the nerve root and beyond into the cochlear nucleus (Ryugo and Rouiller et al., 1988; Brown et al., 1988; Ryugo, 2008). The presynaptic protein filled puncta was highly enriched within the transition zone in the synapse culture highlighting its potential as a dividing zone between the peripheral and central region.

The bundles formed by the axonal processes in the synapse cultures resembled closely the bundle formation of the fibers projecting from the ganglion centrally in acute organ of Corti cultures with intact central target tissue (Fig. 12E-H). In both preparations, the axonal processes extend away from the micro-isolates at a uniform and measureable distance before they change trajectory (compare Fig. 12A to Figs. 12E-F).

[Figure 12]

Adding to the observations of the de novo growth from Thy1-YFP mice, we also explored tissues from CBA/CaJ mice live, at the time of plating the synapse culture (Fig. 13A) and fixed, after 17div (Fig. 13B). This example of an isolated apical organ of Corti micro-isolate (Fig. 13A, dotted lines) plated near an apical neuronal explant with both processes excised (Fig. 13A, arrowheads) showed the proximity of the two tissues and the truncated axonal processes. By comparison, this same culture observed after 17div has elaborated extensive axonal processes, reiterating the attributes previously described: uniform direction, bundle formation, anti-synaptophysin immunolabeling, and fiber trajectory changes that define a distinct transition zone

(Fig. 13B).

Although not always evident, due to the extensive axon bundling often observed in these synapse cultures, the parent branch tends to bifurcate past the transition zone (Fig. 13D). A length measurement from 11 cultures was obtained measured from the cell body to its bifurcation pass the transition zone (Fig. 13E). Therefore, the parent axon bifurcation appeared to be elaborated well below the transition zone in this and many other cultures. The averaged axon length measurements from matched apical and basal synapse cultures were not significantly different.

[Figure 13]

The axon processes elaborated above and below the transition zone *in vitro*, thus, have distinct characteristics that appear to reiterate some of their properties *in vivo*. The directionality and axon bundling compared to that of the intracochlear axons that compose the PNS division of the axon, while the elaboration of synaptophysin and the parent fiber bifurcation at or beyond the transition zone were consistent with characteristics that typify the CNS division of the axon. In order to test the intriguing possibility that the different divisions of the axon bundle were re-formed *in vitro*, we asked whether the length of the transition zone relative the neuronal soma might be altered systematically with tonotopic location. One might expect, for example, that if the PNS portion of the axon was elaborated above the transition zone that it would be longer for matched apical synapse cultures than basal ones due to the greater distance of the apical neuronal somata from the IAM than the basal neurons.

In order to carry out these experiments we compared the distance that the axon bundles traveled from the spiral ganglion neuron explant to the transition zone for matched (Fig.14A) apical, (Fig.14B) middle, and (Fig.14C) basal synapse cultures. In these cultures we did indeed find that a gradient of lengths were reiterated and matched expectations for the relative intracochlear (PNS) distances. The axon projection lengths from the matched apical co-cultures (Fig. 14A, D) were significantly longer (956.6μm ± 52.6, n=30; $p<0.01$) than the matched basal co-cultures (387.3μm ± 51.1, n=22; Fig.14C, D), while the matched middle co-cultures displayed

intermediate lengths (715.8μm ± 61.5, n=13; Fig. 14B, D).

If the length of the axonal processes to the transition zone represents the peripheral axonal lengths, then one might expect this to be highly regulated by the peripheral target tissues, since it is the distance from the organ of Corti to the IAM that determined the PNS portion of the axon. By employing mixed synapse cultures we found that this was indeed the case. Basal neuron explant axonal processes increased significantly in length to the transition zone when paired with an apical organ of Corti micro-isolates (911.8μm ± 41.7, n=10; $p<0.01$) compared to matched basal co-cultures (Fig. 14D). The opposite occurred with the apical neuron explants axon length when paired with a basal organ of Corti micro-isolates it decreased (508.0μm ± 63.4, n=10; $p<0.01$) compared to matched apical co-cultures (Fig. 14D). Thus, this robust phenotype was highly regulated by the target tissue and clearly reflected the intracochlear distances observed *in vivo (*Liberman and Oliver, 1984, Fekete et al., 1984*)*. Liberman and Oliver demonstrated the axon length of apical neurons range from 6000μm to 7000μm whereas, the length of the basal neurons range from 3000μm to 4000μm.

[Figure 14]

An important specialization that would further distinguish the regions above and below the transition zone as either PNS or CNS is myelination. In the peripherally-isolated cultures, the peripheral axonal fibers were myelinated exclusively by Schwann cells then one might expect the myelination to occur exclusively in the PNS portion of the axon since only peripheral satellite cells would be expected to be found in these cultures. In order to carry out these experiments we used myelin basic protein because it can detect both Schwann cell and oligodendrocyte myelination (Boggs, 2006; Toesca, 1996). While myelination was apparently sparse in these cultures (Fig. 15A), the anti-MBP antibody labeling that we did observe was limited to only those fibers above the transition zone, in the putative PNS portion of the axon (Fig. 15B; top box in A). Within the transition zone (Fig. 15C; middle box in A) and beyond (Fig. 15D; bottom box panel A) in the region the fibers will eventually bifurcate no anti-MBP has, as yet, been detected. Even the examination of a synapse culture incubated for longer lengths of time (22 div) enabling more

fibers to be myelinated demonstrated anti-MBP antibody labeling localized to only fibers above the transition zone. This result showed, remarkably, myelination further defined these specialized regions in the synapse culture. Thus, it appears that the peripheral target tissue plays a strong role in regulating the ratio difference between the peripheral and central portion of the axonal processes.

[Figure 15]

To determine the role that neurotrophins play in regulating the putative PNS/CNS axon ratios, we evaluated the impact of supplementing cultures with neurotrophins. We evaluated matched synapse cultures with exogenous application at 5ng/ml of NT3, or 5ng/ml of BDNF with and without function blocking BDNF and NT3 antibodies. These results showed the axon distance from the explant to transition zone was oppositely regulated by BDNF and NT3 depending on cochlear location. Apical neuron explants paired with apical organ of Corti micro-isolates supplemented with BDNF (Fig. 16A) reduced the axon projection length (738.4μm ± 51.0, n=8) compared to control (955.6μm ± 52.6, n=30), which was significantly different (p<0.05; Fig.16A). The axon length of the matched apical co-cultures was oppositely regulated with the application of anti-BDNF (1264.0μm ± 86.5, n=5) and was significantly different from control (p<0.05). The matched apical co-cultures with the application of NT3 shortened (875.6μm ± 64.8, n=9) or with anti-NT3 elongated (1114.7μm ± 72.8, n=3; Fig.16A) the length measurement even though it was not significantly different when compared to control. Basal neuron explants paired with basal organ of Corti micro-isolates with either BDNF or NT3 both increased the axon projection length. Matched basal co-cultures with BDNF (695.8μm ± 70.6, n=7) or NT3 (701.0μm ± 89.6, n=8) when compared to control (387.3μm ± 51.1, n=22) was significantly different (p<0.01; Fig.16A). The addition of anti-BDNF (334.1μm ± 89.8, n=3) or anti-NT3 (360.4μm ± 32.1, n=4) to matched basal co-cultures when compared to control showed no differences in axonal projection length. The middle neuron explants paired with middle organ of Corti micro-isolates with NT3 (566.4μm ± 108.1, n=4) or BDNF (929.3μm ± 138.3, n=4; Fig. 16A) when compared to matched middle co-cultures the axon projection length didn't significantly change. Therefore, both neurotrophins act

differently depending on location in the apical region BDNF and NT3 reduced the axon projection length while in the basal region both neurotrophins enhanced axonal projection length.

[Figure 16]

Since neurotrophins have a similar effect on axonal projection length within each specific frequency region, we reasoned that its effects may be mediated though the same receptor, thus implicating $p75^{NTR}$. While $p75^{NTR}$ distribution was expected to be low or absent in the spiral ganglion (Sato et al 2006; Tan and Shepherd, 2006; Provenzano et al., 2011), we anticipated that it may act through non-myelinating Schwann cells or other satellite cells resident in the synapse cultures. Immunocytochemical labeling with anti-$p75^{NTR}$ antibody did show that immunostaining was present throughout the culture (Fig. 17A), with little or no labeling in the neurons (Fig. 17B,C) or the axonal processes (Fig. 17D). Small bipolar cells resembling non-myelinated Schwann cells (Whitlon et al., 2009) were the predominant cell type labeled with the $p75^{NTR}$ antibody. Consistent with the localization of anti-MBP antibody (Fig. 15A) on the putative peripheral processes the anti-$p75^{NTR}$ tended to align with and surround the axonal fibers (Fig. 17E; top box in A). Once the fibers extended beyond the transition zone, the anti-$p75^{NTR}$ antibody labeled cells no longer associated with the anti-β-Tubulin antibody labeled axonal processes (Fig. 17F,G; middle and bottom boxes in A) This feature was consistent with observations of anti-MBP antibody and further defined the two regions above and below the transition zone.

[Figure 17]

Because $p75^{NTR}$ distribution is pervasive in the synapse cultures, we hypothesized that this receptor alone, and in combination with others (Wang et al., 2002; Nykjaer et al., 2004; Mi et al., 2004), may provide regulatory pathways for more than one function. Our initial approach, however, was to determine whether any of the neurotrophic effects on length was altered in the presence of function blocking anti-$p75^{NTR}$ antibody. We investigated the effects of BDNF on matched apical synapse cultures showed that the length decrease mediated by BDNF were blocked in the presence of anti-$p75^{NTR}$ function blocking antibody (Fig.18). Further, we noticed a slight increase in length in the culture in which anti-$p75^{NTR}$ function blocking antibody was supplemented without BDNF. While additional work must be carried out to expand to matched

basal synapse cultures, these observations were consistent with the idea that neurotrophins were capable of modulating the ratio of PNS/CNS axonal regions through $p75^{NTR}$.

[Fgure 18]

While neurotrophins do have an impact on regulating axonal length, they do so by working in opposition to the influence of the micro-isolate. For example, BDNF opposes the normally long distance in matched apical synapse cultures from the neuron explant to the transition zone, while BDNF and NT3 oppose the normally short distance reiterated in matched basal synapse cultures. Thus, the role of the mature neurotrophins, through $p75^{NTR}$, appeared to modulate the regulatory mechanisms resident within the organ of Corti micro-isolates. In order to expand this study to other mechanisms of control, we next evaluated Sortilin, a potential receptor binding partner of $p75^{NTR}$ (Nykjaer et al., 2004; Teng et al., 2005), which together act as a high affinity receptor for proneurotrophins (Teng et al., 2010).

Previous work showed that Sortilin and $p75^{NTR}$ were co-expressed in neonatal spiral ganglion neurons (Tauris et al., 2011). Our examination, consistent with these observations, revealed anti-Sortilin antibody labeling in spiral ganglion soma (Fig. 19G). However, we also found anti-Sortilin antibody immunolabeling of non neuronal cells that did not overlap with the $p75^{NTR}$ labeling of cells having non-myelinating-like Schwann cell profiles (Fig. 19A). A small bipolar cell resembling a non-myelinating Schwann cell surrounding the axon fiber beneath a layer of Sortilin labeled satellite cells (Fig. 19E). The Sortilin labeled satellite cells were predominantly localized beneath the $p75^{NTR}$ labeled non-myelinating Schwann cell profiles (Fig. 19B, top box in A; Fig. 20A-D). Moreover, this profile ended at the transition zone and the anti-Sortilin antibody labeling was exclusively localized in the satellite cells that did not penetrate into the transition zone (Fig. 19C, middle box in A; Fig.20 E-H). Below the transition zone the immunolabeling of anti-Sortilin labeled cells was not present and the axonal processes were not aligned with the $p75^{NTR}$ labeled putative Schwann cell profiles (Fig. 19D, bottom box in A; Fig. 20I-L). This abrupt transition essentially delineates the transition zone that had been already identified by different criteria.

[Figure 19,20]

Non-overlapping anti-Sortilin and anti-$p75^{NTR}$ antibody immunolabeling in non-neuronal cells suggested that these two receptors act independent. However, our immunolabeling of the synapse cultures indicated within the spiral ganglion neuronal somata these receptors maybe present. Different studies reported $p75^{NTR}$ present (Sato et al., 2006; Schecterson and Bothwell, 1994) or absent (Tan and Shepherd, 2006; Provenzano et al., 2011), or only present after injury (Tan and Shepherd, 2006; Provenzano et al., 2011) in spiral ganglion neurons. We do observe some indication that $p75^{NTR}$ may be localized to these cells at relatively low levels (Fig. 19F-I), thus permitting receptor-receptor interactions. Further studies are needed to examine the effects of anti-Sortilin function blocking antibodies, which may reveal the significance of these complex receptor interactions. Nevertheless, it was clear from the specific patterns of Sortilin labeling that this molecule clearly delimits the regions above and below the transition zone.

Chapter III: Spiral Ganglion Process Branching Patterns

The spiral ganglion neurons without its target tissue present are able to extend its processes for a long distance and bifurcate. Intrinsic and extrinsic signals can potentially mediate the distinct outgrowth patterns observed in our synapse cultures. In order to determine the hair cell micro-isolates contribution mediating the outgrowth pattern in the synapse cultures. We need to examine, the growth patterns in neuronal explants, without the presence of their target tissues.

Type I spiral ganglion neurons typically have a classic bipolar phenotype with processes projected from each side of the cell body (Fig. 21A;star), which extend for 100's of microns before the parent axon bifurcates (Fig. 21A;arrow, arrowhead). Type II spiral ganglion neurons have a pseudomonopolar phenotype also elaborated long processes before branching (Fig. 21B, arrowhead) or forming terminal specializations (Fig. 21B, arrow). The hair cell region was known when the explants were cultured. Measurements of the central-projecting axons from the spiral ganglion cell body to the first bifurcation showed no differences between apical and basal neurons (Fig. 21C), which were consistent with measurements *in vivo* (Fekete et al., 1984).

[Figure 21]

While individual neurons have similar parent axon lengths, we found that there were recognizable differences in apical and basal outgrowth patterns. Postnatal spiral ganglion neuron apical explants examined at 10 div elaborated neuronal processes (Fig. 22A), that projected from the cell bodies extending in multiple directions followed a relatively straight trajectory for a distance (Fig. 22B) before branching or changing its trajectory, often by looping back toward the somata (Fig. 22C). In the base the neuronal processes projected from the cell body extended only for short distances before abruptly reversing direction, forming tight loops (Fig. 22D-F).

[Figure 22]

These subtle differences in axon projection patterns were amplified when explant cultures were supplemented with either BDNF or NT3 at 5ng/ml. In the apex with either application of BDNF (Fig. 23A-C) or NT3 (Fig. 23D-E) the peripheral-orientated axons formed small, tight bundles (Fig. 23B, F), which eventually separate towards the end of the process before bifurcating. The central-orientated axons formed bundles that extend uniformly for a distance before they overlap with other neuronal processes (Fig. 23C). The apical neuronal explant supplemented with NT3 the centrally orientated axons extend uniformly for a distance, and formed very prominent crossing patterns (Fig. 23E).

[Figure 23]

In the base with the application of BDNF (Fig. 24A) or NT3 (Fig. 24D) the branching pattern for a base culture does not change. Both neurotrophins just exaggerate the trajectory pattern observed in the basal explant with the increased survival just as observed with NT3. Our findings showed that both explant cultures showed the neuronal processes directed toward the peripheral region formed tight loops, which overlap forming a circular trajectory (Fig. 24B,E) similar to the control cultures. In both BDNF and NT3 supplemented explant cultures only protracted centrally-projecting processes are observed before they overlap. Again, this is in distinction from the synapse culture in which both BDNF and NT3 promote elongated process lengths from the neuronal soma to the transition zone.

[Figure 24]

The presynaptic protein, synaptophysin, was distributed throughout the axonal fibers and the neuronal cell bodies in both apical and basal explant cultures. Furthermore, this random distribution of synaptophysin throughout the axonal fibers does not change with the application of either BDNF (Fig. 23A, 24A) or NT3 (Fig. 23A, 24D). No strict pattern of synaptophysin labeled puncta was localized in either of the processes orientated in the central or peripheral directions. This is in contrast to the organ of Corti micro-isolate co-cultures (Fig. 12A, 13B-C) that showed a strict synaptophysin distribution pattern localized in the cell body, the putative peripheral processes has very little through the extent of the fiber bundle tract, the transition zone was highly enriched with synaptophysin filled puncta, and then synaptophysin labeled puncta were

distributed in fibers throughout the region below the transition zone. Thus, this pattern of synaptic protein distribution was influenced by the hair cell micro-isolates regardless of which frequency region it was isolated from. Synaptophysin distribution was not the only difference between the explant and the synapse cultures. The structural organization of the synapse cultures axonal fiber trajectory was highly organized, uniform, and followed a strict phenotypic pattern between the peripheral and central region. When compared to the explant cultures the pattern of fiber trajectory does not follow a strict organizational pattern. Thus, without its peripheral targets present the organizational pattern observed in the synapse cultures was not observed in the explant neuronal cultures.

DISCUSSION

A general principle is the structure of neuron determines its function. In the brain, neurons come in different shapes and sizes. The elaborate extensively branched fan shaped dendritic tree of the Purkinje cells in the cerebellum to the triangular cell body shape of the pyramidal cells in the cerebral cortex. The shape, length, and size of a neuron determine how information is processed, received, and sent to other neurons.

Spiral ganglion neurons have a bipolar shape with a peripheral axon that innervates the hair cells in the organ of Corti and a central axon that project into the cochlear nucleus. This phenotype places the cell body within the action potential conduction pathway and profoundly impacts signal transmission. The complexity of the structural design of the spiral ganglion is due to its tonotopically graded features. These different structural properties correspond to spontaneous firing rate of spiral ganglion neurons and threshold differences. This may contribute to the heterogeneous patterns observed in the different phenotypic properties of the spiral ganglion. This current study provides evidence that specific morphological features such as soma area and axon length is controlled by position dependent signals from the organ of Corti.

Soma Area Regulation

We characterized two structural proteins within the spiral ganglion. The microtubule-associated protein 2 (MAP2) is a dendritic marker that is localized within the processes for limited distances flanking the cell body. Ankyrin G (AnkG) is a cytoskeletal scaffold protein that localizes other proteins such as ion channels, cell adhesion molecules, extracellular matrix molecules, and cytoskeletal scaffolds to the nodes of Ranvier (Rasband et al., 2010). We demonstrate anti-MAP2 labeling is adjacent to anti-AnkG this configuration pattern defines the nodes that surrounds the cell body. The pattern observed is similar to the *in-vivo* measurements of the non-uniform nodal distance that varied from cell to cell (Robertson, 1976). These spike generators surrounding the cell body enable reliable propagation of an action potential at regions of impedance mismatch such as the cell body. The different ion channel composition of $Ca_v2.2$ and HCN4 show similar staining patterns defined by the MAP2. The labeling highlights the soma

area as electrophysiological important. This characterizes the nodes adjacent to the cell body enabling the action potential to pass through the soma to reach its targets in the central nervous system. A consequence of the cell body being within the conduction pathway is it can act like a frequency filter that limits the passage of high frequency signals (Mellon and Kennedy, 1964;Roberston, 1976).

The size difference between apical and basal neurons is demonstrated utilizing three different preparations: the acute postnatal sections, gangliotopic culture, and the explant culture. This variation in soma size has been characterized in adult animals (Liberman and Oliver,1984; Nadol et al., 1990; Echteler and Nofsinger, 2000). In the gangliotopic and the explant cultures, we observed heterogeneity of soma area within each specific frequency region. Despite the variation within each frequency region there is a statistical difference between the cell size of neurons localized in the extreme apical and basal regions.

This distribution of cell size did not conform to a linear pattern. In contrast, to the graded features observed in the cochlea regulated by BDNF and NT-3 these neurotrophins have an oppositely graded distribution patterns (Fritzcsh et al., 1997; Schimmang et al., 1997; Schimmang et al., 2003; Sugawara et al., 2007). We show soma area has a different regulatory mechanism compared to previous finding examining the firing patterns (Adamson et al., 2002) and synaptic protein distribution (Flores-Otero et al., 2007) in spiral ganglion neurons. Our findings demonstrated no significant changes to basal neuronal size at 10ng/ml while apical neurons only reduced in size. This revealed BDNF doesn't change cell size of either apical or basal neurons but just enhanced the differences between the two neuronal populations. This is the opposite to the regulation of the synaptic protein distribution and voltage-gated ion channel composition such that, features in the base are mediated by BDNF. We predicted we could increase soma area with the application of BDNF. However, apical soma area reduced in size and basal soma area is not affected. We evaluated NT-3 at a range of concentrations on apical and basal neurons only the apical neuronal area is significantly reduced. In comparison, to previous findings that demonstrated features in the apex are mediated by NT3. We observed a decrease in apical area but no change in the basal soma area. Thus both BDNF and NT3 only enhanced the differences

between apical and basal neurons. The combination of different concentrations between BDNF/NT3 revealed a similar pattern in soma area correlating to both BDNF and NT3 separately. This reduction in soma area in only the apical neurons from both BDNF and NT3 is an unexpected result. A reduction in size is usually thought of being unhealthy however, an increase in survival for BDNF, NT3, and BDNF/NT3 indicating the health of the neurons. We observed that both neurotrophins preferentially enhanced apical neuronal survival and reduced apical soma size. Overall, soma area is differentially regulated from the graded properties already characterized within these neurons. Since BDNF, NT3, and combination of both all show the same effect on cell size.

We used the organ of Corti micro-isolates bioassay to examine the endogenous source of trophic factors. Our approach analyzed the effect of the hair cells and their accompanying supporting cells on cell size. We show that cell size between tonotopic regions is retained within the matched co-cultures for apical, middle, and basal organ of Corti micro-isolates paired with apical, middle, and basal neurons. Our findings showed that micro-isolates taken from different cochlear regions when mix-and-matched with spiral ganglion neurons could alter soma area. The tropic factors secreted from the micro-isolates neurotrophins BDNF and NT3 are prominent in the postnatal cochlea. Both neurotrophins are found to mediate the change in size with anti-function blocking antibodies within the mix-and-matched cultures. These results show that within the context of the micro-isolates, the soma size can be predictably altered.

The exogenous application of BDNF and NT3 showed that these two neurotrophins are likely not the only regulators. This most likely reflects the complexity of regulating the neuronal morphological features, such as soma area, axon length, and myelination, which are critically important in assuring accurate and reliable electrophysiological transmission. To better understand the role of a cofactor in mediating soma area we evaluated the apical, middle, and basal co-culture with apical, middle, and basal neurons. Our findings showed only basal neurons may require a co-factor to alter size since the apical or middle neurons didn't change in size with the application of NT3 or BDNF. The mechanism that mediates this change in size could be supported by the expression of neurotrophins from the spiral ganglion neurons. It has been

reported that cultured spiral ganglion neurons express the neurotrophins BDNF and NT3 (Hansen et al., 2001). It is possible that the neurotrophins expressed by the spiral ganglion neurons could interact act with the trophic factors secreted by the hair cells to mediate this change in size that cannot be reproduced in the explant cultures.

We demonstrate that the response of apical or basal neurons to BDNF and NT3 is the same; the absolute sizes between the neurons are retained. These two neurotrophins only serve to enhance the original size of the neuron by either decreasing apical neuron somata size or slightly increasing basal neuron somata size. Compared to previous findings, different mechanisms undoubtedly regulate soma size, that likely are mediated by the p75 receptor since BDNF and NT3 have similar effects on apical and basal neurons for soma area.

PNS/CNS ratio of the axonal process

Signals from the organ of Corti induce spiral ganglion neurons to acquire axon profiles that have PNS-like and CNS-like characteristics. When neuronal explants are co-cultured with micro-isolates taken from different regions of the organ of Corti. We examined the role of the peripheral targets in regulating the difference in the ratio length between the putative peripheral and central regions. We highlighted different molecular and structural features that distinguish these regions as peripheral and central. Furthermore, we show the ratio differences in axon length between these two regions can be modulated by BDNF and NT3 through the p75 receptor.

The outgrowth pattern of the spiral ganglion neurons when co-cultured with organ of Corti micro-isolates using Thy-1 mice showed the *de novo* growth of the axonal processes. One process that re-innervates the hair cell region while the other process projects from the cell body and extends for a distances and branch. The axonal process projecting away from the cell body shows reminiscent similarities to bundle patterns observed *in vivo*. We demonstrated the axonal processes innervating the hair cell region majority of them loop away from the hair cell micro-isolates. This suggests repulsive cues could be coming from this region. Many repellants such as Slits (Battisti and Fekete, 2008), Semphorins (Chilton and Guthrie, 2003), and Ephrins (Pickles et. al., 2002) have been characterized in the mammalian inner ear and could play a role.

Our findings revealed that when an explant of spiral ganglion neurons is co-cultured with organ of Corti micro-isolates. The organ of Corti micro-isolates influence the directional and uniform bundle outgrowth pattern of the axonal processes projecting away from the cell body. We noted certain attributes, which are always consistently found within these co-cultures. The uniform fiber bundles that change orientation once the axonal fibers extends pass a specialized region we refer to as the transition zone. Once the axonal process extend pass this region the processes became unorganized, non-directional and bifurcate in multiple locations. We observed the distribution of the presynaptic protein synaptophysin, which could be used as a marker to distinguish between the region above and below the transition zone. This marker and the different organizational patterns described distinguish the regions above and below the transition zone. This highlights the role of the hair cell micro-isolates in mediating these different features.

We developed a new culture preparation for this study, which is an organ of Corti culture still connected to its central target tissue. This acute culture retains the uniform radial bundle pattern described *in vivo* for the peripheral fibers innervating the hair cells. The axon projecting towards the central direction show distinct bundles formed from different clusters of neurons. This culture distinguishes peripheral from central regions when compared to the organ of Corti micro-isolate co-cultures. Similar features can be observed the bundling of processes projecting from the cell body, the uniformity of the axonal outgrowth, and the change in direction and organization of the axonal processes within the region below the transition zone. This links these separate regions highlighted by differences in synaptic protein distribution and organization of the axonal fibers within the organ of Corti micro-isolates co-cultures in the peripheral and central side.

To examine myelin localization within the organ of Corti micro-isolate co-cultures we used myelin basic protein (MBP), which is expressed by Schwann cells and oligodendrocytes (Boggs, 2006). Our findings utilizing anti-MBP antibody on synapse cultures showed labeling around the putative peripheral axonal processes while no labeling is observed below the transition zone. Since the central target tissue is not present in our co-culture system only the Schwann cells are present in the culture, thus MBP is situated in the correct region. The MBP situated only on the peripheral portion and other phenotypic attributes already described such as synaptophysin

levels and the structural organization is localized to this same region above the transition zone. These different features help to distinguish this portion of the process from the region below the transition zone. Since not many processes are myelinated in culture to verify that only the Schwann cells are present and myelinating the peripheral portion of the process. Additional experiments completed at longer in vitro time periods verified the labeling of the myelin basic protein is present only in Schwann cell myelin and not the oligodenoctye myelin.

We showed that the organ of Corti micro-isolates co-cultures potentially regulate the ratio difference between the peripheral and central length. The putative peripheral process that differs with cochlear location reflects the intracochlear axon, which describes the peripheral process segment *in vivo*. Our findings show a graded distribution of the putative peripheral axonal processes when organ of Corti micro-isolates taken from different tonotopic regions are mix and matched with spiral ganglion neurons. This showed that frequency-dependent regulation by the organ of Corti micro-isolates has a profound effect on spiral ganglion neuron axonal morphology. To understand the role of neurotrophins in regulating the described ratio differences between the peripheral and central region. Two neurotrophins are examined, BDNF and NT3, which are highly expressed in the developing inner ear. When applied to the matched apical, middle, and basal co-cultures both BDNF and NT3 had differentially effects on mediating the putative peripheral process depending on frequency region. However, our findings showed that when these neurotrophins when exogenously added to the organ of Corti co-cultures the process lengths to the transition zone is modulated.

Our findings revealed that the Sortilin positive cells are localized to different types of non neuronal cells. One type of cells showed a resemblance to mesenchymal cells observed by Coate and colleagues utilizing an antibody against Pou3f4 protein. This transcription factor belongs to the POU domain of proteins. The expression of the Pou3f4 protein is mainly localized to the otic mesenchymal cells. This study showed that the organized radial bundle pattern of the peripheral axons could be disrupted when the Pou3f4 protein is knocked down. The mechanism identified to regulate the radial bundle pattern is the Pou3f4 protein localized in the otic mesenchymal cells directly activates the EphA4 receptor situated on the surface of these cells.

The EphA4 receptor binds to the ephrin-B2 localized to the axons leading to the bundling of the peripheral fibers (Coate et al., 2012). This shows the ability of the otic mesenchyme to have an organizational effect on the peripheral axonal fibers using a paracrine signaling pathway. In comparison, to our findings, anti-Sortilin antibody staining is localized to mesenchymal-like cells that are mainly localized to the peripheral region. This localization mainly above the transition zone is another marker to help define this region as peripheral. Sortilin shows a clear demarcation line that clearly distinguishes the peripheral from the central region. The identity of the Sortilin-positive cells is not known. Preliminary experiments of isolated regions of the organ of Corti micro-isolates compared to neuronal explants show Sortilin-positive cells are only present within the hair cell micro-isolates cultures. These cells maybe getting cues from the organ of Corti to organize the transition zone since these cells are the strongest indicator of this boundary.

In addition, to this marker and in combination with the previous findings already described shows that the regions above and below the transition zone are characterized by specific attributes that define these two regions. These findings highlight the biological differences indicated by these molecular makers described above showing a distinction in the region above and below the transition zone.

The ratio differences are further highlighted with the $p75^{NTR}$ which labels the Schwann cells showing an alignment with the axonal fibers in the peripheral region. Studies showed that $p75^{NTR}$ expression is localized to the spiral ganglion in neonatal animals (Tauris et al., 2011) but low and limited levels expression levels in the postnatal spiral ganglion neurons (Tan and Shepherd, 2006; Provenano et al., 2011, Sato et al., 2006). The $p75^{NTR}$ is found to be in glial and satellite cells (Liu et al., 2012) and the expression levels is increased in injured cells (Tan and Shepherd, 2006; Provenano et al., 2011). We noted the partial ensheathment of the nerve fiber observed with anti-$p75^{NTR}$ immunoreactivity at the outer most portion of the axon resembled the myelin sheath found when labeled by anti-MBP antibody. The $p75^{NTR}$ has been shown to act as a positive modulator of myelination (Cosgaya et al 2002). Blocking $p75^{NTR}$ in Schwann cells/dorsal root ganglion co-cultures showed that the two myelin proteins, myelin-associated glycoprotein (MAG) and P0 accumulation are inhibited. Two neurotrophins BDNF and NT3 are shown to

modulate the accumulation of these myelin proteins, BDNF increases and NT3 decreases myelin protein accumulation (Cosgaya et al 2002). They showed BDNF acts like a positive modulator through $p75^{NTR}$ and NT3 acts like a negative modulator of these myelination proteins. Myelin protein is enhanced by BDNF and decreased by blocking trkB receptor in the Schwann cell/dorsal root ganglion co-cultures, while no effect is observed from $p75^{NTR-/-}$ cultures (Cosgaya et al 2002). NT3 inhibited and blocked the receptor enhanced myelination in both wildtype and $p75^{NTR-/-}$ cultures (Cosgaya et al 2002). These results suggest that $p75^{NTR}$ and Trk receptors have the opposite effects on myelination.

Therefore, the link between the axon and Schwann cell mediating axon morphology could be the $p75^{NTR}$ receptor because it is expressed on the Schwann cells. This receptor may be regulating the association between the Schwann cell and the axon therefore, regulating the ratios between the peripheral and central portion of the process. Our findings add another element to this idea that Sortilin maybe the substrate that mediate the interaction between $p75^{NTR}$ and the axon thus, mediating the ratio differences between the peripheral and central region. Our $p75^{NTR}$ blocker experiments supports this hypothesis that $p75^{NTR}$ may be the regulatory element behind the ratio differences. Our findings showed when the $p75^{NTR}$ is blocked with the addition of BDNF the peripheral portion of the process elongated thus, BDNF is blocked from binding to the $p75^{NTR}$. The $p75^{NTR}$ seems to be an important mediator of regulating axon bundle ratio differences between peripheral and central portion of the axonal process. To understand the role $p75^{NTR}$ play in mediating this process we need to understand what receptor is associated with the $p75^{NTR}$. In addition to trkB and trkC high affinity receptors, $p75^{NTR}$ can complex with Nogo receptor (NgR) and Lingo-1 receptor to signal through the RhoA pathway (Ibanez and Simi, 2012). A future experiment will need to be completed to determine the distribution pattern of each of these receptors in our culture to characterize whether they specifically associate with the $p75^{NTR}$.

The transition zone is the region in our organ of Corti micro-isolates co-cultures that separates the peripheral and central processes. It is highlighted in our findings in multiple examples showing large puncta densities of synaptic protein distributed across this area. *In vivo*, synaptic like specializations were observed for collateral branches from type I and type II

neurons near the Schwann-glial border (Brown et al., 1988). In our findings, the central processes are tightly bundled as its pass though the transition zone then the fibers begin to separate and project over one another to form a crisscross pattern. In the animal, the central axonal fibers that extend to the cochlear nucleus which bundle together to form the eight nerve and branch according to its innervation position in the organ of Corti (Appler and Goodrich, 2011; Ryugo, 1992; Nayagam et al., 2011; Fekete et al., 1984). Our cultures do not have their central targets present but the fibers bifurcate without forming the orderly pattern observed *in vivo*. Other features present in our culture similar to *in vivo* attributes such as collateral branches that arise from the parent branch and are general very thin in comparison to the main axon that can form secondary branches (Fekete et. al., 1984;Brown et al., 1988). We showed examples of rudimentary terminal ending that have been observed at the end of the process after the bifurcation in our organ of Corti micro-isolates co-cultures.

Our findings suggest that our co-cultures elaborate a complex system in which the peripheral and central axonal regions are delineated. This is established by the unique distribution patterns of different biological markers, such as $p75^{NTR}$, Sortilin, synaptophysin, and MBP. Also the distribution of structural properties such as the organization of the axonal fibers that reflect tonotopic intracochlear axon length and the branching and terminal endings, which are localized mainly below the transition zone.

Explant Branching Patterns

The different axonal branching patterns observed between apical and basal neurons without the presence of its peripheral targets can be mediated by intrinsic or extrinsic mechanisms. First, an intrinsic signal that could mediate the differences in the pattern observed between apex and base explant cultures. The transcription factor involved in mediating different aspects of phenotypic properties should have a differential expression pattern between apical and basal neurons. The transcription factor MafB is expressed in the spiral ganglion neurons in a graded pattern. The expression of this transcription factor is at E13 its strongly expressed in basal neurons and weak in the apical region this pattern is observed at postnatal age six (Lu et al.,

2011). The MafB is involved in the formation of postsynaptic terminals. In Maf B mutant mice, the post synaptic density fail to develop and reduces the number of synapse formations (Yu et al., 2013). The transcription factor Gata3 is known to activate spiral ganglion specific transcription factors (Appler et al., 2013) such as MafB. This transcription factor Gata3 has been shown to control neurite guidance for the peripheral spiral ganglion neurons innervation patterns, control the timing of neurite extension, and the trajectories within the cochlea. This shows that specific transcription factors found to be exclusively within the cochlea neurons specific to frequency region may have the potential to control different aspects of apical and basal neuron phenotype.

Secondly, extrinsic mechanism that can mediate the differences in the branching patterns between the apical and basal neurons is the non-neuronal cells. Satellite cells are localized along with the spiral ganglion neurons that may provide guidance to axonal fiber extension. Puligilla and colleagues, show interruption of FGF signaling in the supporting cells triggers the disorganization of fibers projecting to the outer hair cells (Puligilla et al., 2007). This population of cells has the potential to influence the innervation patterns in the spiral ganglion neurons. The contribution of the supporting cells and Schwann cells provides to spiral ganglion neurons is not known. However, if the supporting cells are differentially regulated between the apical and basal neurons it has the potential to influence the branching patterns observed. Nonetheless, since Schwann cells and supporting cells share many characteristics such as glial markers (Morris et al., 2006). This makes it hard to identify the specific contribution of each population of cells may contribute to spiral ganglion development (Appler et al., 2011).

Guidance molecules are known to be expressed in non sensory cells of the inner ear have both inhibitory and attractive signals that can mediate neurite fiber projection pattern in neurons. Guidance molecules such as Ephrins can provide attractive and repulsive cues (Baltisti and Fekete, 2008; Pickles et al., 2002). In the mouse, the ephrin-B2 and ephrinA4 both have an inhibitory influence on postnatal spiral ganglion axonal growth *in vitro* (Bianchi and Gray 2002). It is shown that ephrins have a tonotopic pattern from base to apex gradient in the cochlea of birds (Siddiqui and Cramer, 2005). The graded pattern of this guidance molecule could be a way to affect the different apex and base axonal patterns. This could be one of other guidance

molecules that are working in complement with one another to develop the pattern observed between apical and basal neurons. Guidance molecules are also in the extracellular matrix, which provides a substrate for the axon and can facilitate the fiber as it travels to its target destination (Whitlon et al., 1999a,b). The extracellular matrix can play a role in mediating the axonal pattern between the apex and base explant cultures.

SUMMARY

In conclusion, the regulation between the soma area and the ratio of the axonal process between the peripheral and central region both show a different regulatory mechanism when compared to previous findings of the lab. We showed previously that features in the apical region can be unregulated by NT3 and down-regulated with BDNF, regardless of cochlear location. The opposite is shown for features in the basal region can be up-regulated by BDNF and down-regulated with NT3 examining synaptic protein distribution. These findings presented herein show that BDNF and NT3 have a differential affect that is dependent on cochlear location. We show that within a specific frequency region BDNF and NT3 have a similar phenotypic effects. Consistent with the idea that the p75 receptor may be involved in regulating multiple phenotypes; it has been shown that this receptor does not only work alone, it also works in complex with other receptors to facilitate a vast array of biological responses. The next step would be to examine the other identified co-receptors that can bind to p75 such as trkB, trkC, LING0-1, Nogo-66 and orchestrate a biological response.

Experiments designed to investigate the regulatory mechanisms that orchestrate outgrowth patterns, has only just begun, have already revealed complex mechanisms that appear to also involve myelination, cell-to-cell associations, and intracellular receptor distributions. Thus, by having developed an accessible culture system to investigate mechanisms that control the putative PNS axonal regions from the CNS ones, we may have the opportunity to elucidate some of the fundamental principles that define the two different specific regions of these hybrid PNS/CNS primary auditory afferents.

FIGURE LEGENDS

Figure 1. The soma and nuclear area was accurately measured using anti-β-tubulin antibody**. A-D**, The soma area of spiral ganglion neurons labeled with anti–β-tubulin antibody resembled the anti-HCN1 antibody which outlined the cell membrane. **A-B,** Spiral ganglion neurons were labeled with (B) anti-HCN 1 antibody (green) and (A) anti-β-tubulin antibody (red). **C,** The merged image from panels (A) and (B) labeled with the yellow dotted and white lines which outlined the soma area measurement from both antibodies. **D,** The scatter plot showed a linear distribution between anti-β-tubulin and anti-HCN1 from a single experiment. **E-H**, The nuclear area of spiral ganglion neurons corresponded to measurements completed with DAPI and Hoescht dye. **E**, The anti–β-tubulin antibody (red) labeled the neurons and (F) Hoescht dye (blue) labeled the nuclear area indicated by arrowheads. **G,** The merged image from panels (E) and (F). **H,** The scatter plot showed a linear pattern between anti-β-tubulin and the nuclear dye (Hoescht and DAPI) from four different experiments. Scale bar in C applies to A-C and E-G.

Figure 2. In the cochlea, spiral ganglion neurons range in different shapes and cell body sizes. **A**, Bipolar and pseudomonopolar spiral ganglion neurons labeled with anti-microtubule-associated protein 2 antibody. **B**, A type III neuron indicated by the extremely large cytoplasm to nuclear area ratio labeled with anti-β-tubulin. This neuron localized primarily in apical cultures. Scale bar in B applies to A. **C-H,** A range of different soma sizes within each frequency region (C-E**)** apical (F-H**)** basal neurons labeled with anti-β-tubulin. Scale bar in H applies to C-G. **I-J,** Frequency histograms of soma area measurements from a single experiment of apical (I) and basal (J) neurons showed a wide range of cell sizes.

Figure 3. The electrophysiological significance of the cell body demarcated by microtubule-associated protein 2 (MAP2) antibody labeled the cell body and initial processes. **A-B,** The (A) pseudomonopolar and bipolar (B) neurons labeled with anti-MAP2 antibody (red). The MAP2 label was immediately flanked by anti-AnkG antibody (green). Scale bar in A applies to B. **C,** The

plot reflects the molecular configuration of these structural proteins which was correlated with distance (green, AnkG; red, MAP2). **D-F,** A spiral ganglion neuron labeled with (D) anti-MAP2 antibody and (E) anti-HCN4 antibody was distributed within the same intracellular region. The full extent of the processes was labeled with (F) anti-β-tubulin antibody. Scale bar in F applies to D-E.

Figure 4. Different preparations demonstrate consistently soma and nuclear area varies with frequency region. **A-D**, Soma area has a graded distribution pattern. **A,** A low magnification image of a paraffin-embedded tissue section. **B-C,** High magnification images of neurons isolated from the extreme apical and basal regions. **D,** The average soma area measurements from the apex, mid-apex, mid-base, and base regions. The number of experiments localized within the bars, ** represent $p<0.01$, and * represent $p<0.05$ in this and the following figures. The scale bar in C applies to B. **E-H**, The gangliotopic culture, showed soma area had a non linear relationship to tonotopic position. **E.** The low magnification image of a gangliotopic preparation cultured for 4 div. **F-G.** High magnification images of neurons isolated from the extreme apical and basal regions. **H,** The scatter plot of soma area measured from the image shown in E. Soma area was plotted against the percent distance from apex. The line was fitted by eye. The black circles represent the average number of measurements taken from 12 individually spaced circles along the ganglion. The grey circles represent the individual measurements of neurons. Scale bar in G applies to F. **I-J,** Neuronal explants isolated from the extreme apical and basal regions. **K-L,** The average nuclear (K) and soma area (**L**) measurements from eight different experiments. Scale bar in J applies to I.

Figure 5. Apex and base neuronal explants supplemented with different concentrations of brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) retain their same size differences. **A,** Apical and basal spiral ganglion neurons treated with BDNF at different concentrations from 1, 5, 10, 50 and 100ng/ml. The average soma area measurements demonstrated a significant reduction at 10ng/ml in only apical neurons. **D,** The difference in soma area between apical and basal neurons was evaluated at all concentrations. **B,** Apical and basal spiral ganglion neurons

treated with NT3 at concentrations from 0.01, 0.1, 0.25, 0.5,1, 5, 10, 50, and 100ng/ml. The average soma area measurements from apical and basal neurons showed a significant reduction of only apical neurons. **E,** The difference in soma area between apical and basal neurons was evaluated at all concentrations. **C,** Apical and basal spiral ganglion neurons treated with ratios of BDNF/NT3 at the following concentrations: 2.5ng/ml BDNF/7.5ng/ml NT3, 5ng/ml BDNF/5ng/ml NT-3, 7.5ng/ml BDNF/2.5ng/mlNT3, and 10ng/ml BDNF/10ng/ml NT3. The average soma area measurements treated with different ratios of BDNF/ NT3 did not alter soma area. **F,** The difference in soma area between apical and basal neurons was evaluated at all concentrations.

Figure 6. Explant cultures supplemented with different concentrations of BDNF and NT3 increased spiral ganglion neuronal survival. **A,** Apical and basal spiral ganglion neurons treated with BDNF at concentrations from 1, 5, 10, 50 and 100ng/ml. **D,** The difference in survival was evaluated between apical and basal neurons at all concentrations. **B,** Apical and basal spiral ganglion neurons treated with NT3 at concentrations from 0.01, 0.1, 0.25, 0.5 1, 5, 10, 50, and 100ng/ml. **E,** The difference in survival was evaluated between apical and basal neurons at all concentrations. **C.** Apical and basal spiral ganglion neurons treated with ratios combinations of BDNF and NT3 at concentrations of 2.5ng/ml BDNF/7.5ng/ml NT3, 5ng/ml BDNF/5ng/ml NT3, 7.5ng/ml BDNF/2.5ng/ml NT3, and 10ng/ml of BDNF/ 10ng/ml of NT3. **f.** The difference in survival was evaluated between apical and basal neurons at all concentrations.

Figure 7. In the synapse culture, spiral ganglion neurons regenerated their processes and re-innervated the hair cells *in vitro*. **A,** A low magnification image of apical hair cell micro-isolates paired to apical neurons supplemented with 5ng/ml BDNF. The peripheral processes from the cluster of spiral ganglion neurons labeled by anti-β-tubulin antibody (red) re-innervated the hair cells labeled by anti-calbindin antibody (blue). **B,** The high magnification image of the synapses labeled by anti-synaptophysin antibody (green) at the inner hair cells (IHCs) and their afferent terminals.

Figure 8. The soma area of spiral ganglion neurons altered when paired with peripheral target tissue taken from defined regions of the organ of Corti. **A-C,** Spiral ganglion neurons were labeled with anti-β-tubulin. The cultures (A) apical hair cell micro-isolates paired to apical neuronal explant (H(A)N(A)), (C) basal hair cell micro-isolates paired to basal neuronal explant (H(B)N(B)), and (B) middle hair cell micro-isolates paired to middle neuronal explant (H(M)N(M)). **D,** Average soma area measurements from the matched apex, middle, and base neuronal explants paired to matched apical, middle, and basal hair cell micro-isolates. **E-G,** The soma area of neuronal explants was altered when paired to mixed-and-matched hair cell micro-isolates taken from different frequency regions. The cultures (E) basal hair cell micro-isolates paired to apical neuronal explant (H(B)N(A)), (G) apical hair cell micro-isolates paired to basal neuronal explant (H(A)N(B)), and (F) middle hair cell micro-isolates paired to basal neuronal explant (H(M)N(B)). **H**, Average soma area measurements from the mixed-and matched hair cell micro-isolates paired to neuronal explants. A sub-set of co-cultures were supplemented with anti-BDNF and anti-NT3 function-blocking antibodies. Scale bar G applies to A-C and E-F**.**

Figure 9. The exogenous application of BDNF and NT3 to matched apex, middle and base co-cultures demonstrated basal neurons require a co-factor to alter cell size. **A**, Average soma area measurements from apical, middle, base neuronal explants paired to matched apical, middle, base hair cell micro-isolates. The cultures were supplemented with BDNF or NT3 together or separately. Some co-cultures were supplemented with anti-BDNF or anti-NT3 function blocking antibodies. **B,** The difference in soma area was evaluated utilizing apex, middle, and base matched co-cultures.

Figure 10. BDNF and NT3 consistently enhanced survival within each frequency region but the largest enhancement was in the apex. **A,** The average survival measurements from apical, middle, and basal neuronal explants paired to its matched apical, middle, and basal hair cell micro-isolates. The cultures were supplemented with BDNF or NT3 together or separately. Some co-cultures were supplemented with anti-BDNF or anti-NT3 function blocking antibodies. **B,** The difference in survival was evaluated utilizing apex, middle, and basal matched co-cultures.

Figure 11. Two Thy1-YFP mouse lines were utilized to analyze live outgrowth at different times periods. **A**, An organ of Corti culture, the spiral ganglion neurons and their processes were illuminated. **B,** A synapse culture, at initial isolation of the hair cell micro-isolates (dotted line) cultured near a neuronal explant (SGN; arrowheads). **C,** In the synapse culture, the neuronal process elaborated de novo after 12div. **D**, Illustrate the turning of the peripheral process that sometimes occur in the synapse cultures. **E,** The processes extend for long distances away from the neurons in an orderly direction. **F,** The spiral ganglion neuron and its processes were fluorescent. The axon processes bifurcate (G, H) and have rudimentary endings (H, J).

Figure 12. The directional and organized axon outgrowth pattern of spiral ganglion neurons consisted observed within the synapse culture. **A-D**, A synapse cultures were labeled by anti-β-tubulin antibody (red), anti-synaptophysin antibody (green) and anti-calbindin antibody (blue). **A**, A low magnification montage of an apex matched synapse culture with 5ng/ml of BDNF and NT3 showed uniform axon bundle pattern above the transition zone (double arrowheads). The transition zone has a high density of synaptophysin (green) filled punta at and below the transition zone (arrowhead). **B**, The high magnification image exhibited the axon bundle organization and the high density of synaptophysin at and below the transition zone. **C-D**, The high magnification image of the (C) transition zone and (D) below this region from panel A. **E-H,** An acute organ of Corti culture still connected to its central targets. This culture showed the organized innervation and bundle patterns observed in the animal. **F**, High magnification image of an individual bundle in the dotted box in E. The highly organized axon bundles groups projecting away from the spiral

ganglion neurons. **G-H,** High magnification images consisted of cells with structural characteristics of central neurons.

Figure 13. The examination of the spiral ganglion processes elaborated de novo from the neuron explant to the transition zone in CBA/CaJ mice. **A-B,** An apex matched synapse culture (A) demonstrated at initial plating and isolation and (B) after 17div labeled by anti-β-tubulin antibody (red), anti-synaptophysin antibody (green), anti-calbindin antibody (blue). Matched apex synapse culture exhibited uniform and directional bundling of the axonal processes from the explant to the transition zone. **C,** The low magnification image of a matched apex synapse culture demonstrated similar structural organization as in B. **D**, A high magnification of the axon process bifurcating below the transition zone. **E**, The distance from the neuronal explant to the first bifurcation point was not significantly different between the matched apex and base synapse cultures.

Figure 14. The axon length measured from the explant to the transition zone varies with cochlear location **A-C,** Matched (A) apex, (B) middle, and (C) base synapse cultures labeled by anti-β-tubulin antibody (red) and anti-calbindin antibody (blue). **D**, The axon length in mixed and matched synapse cultures was graded with cochlear location. The hair cell micro-isolates was shown to mediate this length measurement. Scale bar in C applies to A-B.

Figure 15. Myelin basic protein was distributed preferentially above the transition zone in the synapse culture. **A**-**D**, A matched apical synapse culture labeled by anti-β-tubulin (green), anti-myelin basic protein antibody (red). The dotted boxes identify the region above, at, and below the transition zone. **B**, The anti-myelin basic protein antibody (red) labeled the myelin surrounding the neuronal process labeled by anti-β-tubulin (green). **C-D**, The (C) transition zone and (D) below no anti-myelin basic protein immunolabeling was located within these regions. Scale bar in D applies to B-C.

Figure 16. In matched apex and base synapse cultures, BDNF and NT3 only modulate the effect of the hair cell micro-isolates on axon length. **A-B,** Matched apical, middle, and base synapse cultures treated with either BDNF or NT3 separately or together at 5ng/ml. The averaged measurements in the apex matched synapse cultures BDNF or NT3 reduced axon length. In contrast, in base matched synapse cultures BDNF or NT3 increased axon length. **B**, The difference between the matched apex, middle, and base synapse cultures demonstrated axon length was differentially regulated depending on frequency region.

Figure 17. In the synapse culture, the anti- $p75^{NTR}$ antibody immunolabeled satellite cells with non-myelinating Schwann cell-like characteristics. The synapse culture was immunolabeled by anti-$p75^{NTR}$ antibody (green) and anti-β-tubulin antibody (red). **A**, A low magnification of multiple images merged together of an apex matched synapse culture. **B-C,** High magnification images of the spiral ganglion neurons **B,** The merged image demonstrated the neurons were not immunolabeled by anti-$p75^{NTR}$ antibody. **C,** The small bipolar cells (green) resembling the non-myelinating Schwann cells formed a halo around the neurons. **D**, An axonal process (red) surrounded by Schwann-like cells (green) labeled by anti-$p75^{NTR}$ antibody. **E-G,** High magnification images of the regions above, at, and below the transition zone. **E**, The Schwann-like cells (green) were in alignment with the neuronal processes (red) above the transition zone (top box in A). **F**, The alignment of the Schwann-like cells with the neuronal processes at the transition zone (middle box in A). **G**, The Schwann-like cells were not aligned with the neuronal processes below the transition zone (bottom box in A).

Figure 18. The $p75^{NTR}$ plays a role regulating axonal ratio length. Apical hair cell micro-isolates co-cultured with an apical spiral ganglion neuronal explant the findings showed the $p75^{NTR}$ blocker inhibited the effect BDNF had on decreasing axon length.

Figure 19. The Sortilin labeled different non neuronal cell populations above the transition zone. **A-I,** The synapse culture was labeled with anti-Sortilin antibody (green), anti-β-tubulin antibody (blue), and the anti-$p75^{NTR}$ antibody against the extracellular domain (red). **A,** A low magnification image of an apical matched synapse culture supplemented with 5ng/ml NT3. The dotted boxes identify different regions within the culture above, at, and below the transition zone. **B,** The anti-Sortilin antibody labeled a different class of satellite cells beneath the Schwann-like cells labeled by $p75^{NTR}$ (top box in A). **C,** The transition zone showed the abrupt labeling of anti-Sortilin antibody at the border of this region (middle box in A). **D,** The region below the transition zone the neuronal processes were not in alignment with anti-$p75^{NTR}$ antibody immnolabeling of Schwann-like cells. The anti-Sortilin antibody immnolabeling was not abundant within this region (bottom box in A). **E,** A neuronal process (blue) surrounded by Schwann-like cell (red) above the punctate labeling of anti-Sortilin labeled satellite cells (green). Scale bar in E applies to B-D. **F-I,** High magnification image of spiral ganglion neurons labeled by (F) anti-β-tubulin antibody, (H) anti-$p75^{NTR}$ antibody, and (G) anti-Sortilin antibody, and the (I) merged image from panels F-H. Scale bar in I applies to F-H.

Figure 20. Sortilin preferentially labeled the satellite cells above and at the border of the transition zone. **A**-**L**, The regions (A-D) above, **(**E-H) at, and (**I**-**L**) below the transition zone labeled with anti-β-tubulin antibody (blue), anti-Sortilin antibody (green), and anti-$p75^{NTR}$ antibody (red). **A**-**C**, The axons labeled with anti–β-tubulin were aligned with the bipolar cell-like profiles labeled with $p75^{NTR}$ were above the layer of satellite cells labeled with anti-Sortilin. **D**, The merged image from panels A-C. **E**-**G**, The satellite cells labeled with anti-Sortilin formed a distinct border beneath the processes labeled with anti-β-tubulin at the transition zone. **H**, The merged image from panels E-G. **I**-**K,** The satellite cells labeled with anti-Sortilin were not prominent beneath the axons labeled with anti-β-tubulin. **L**, The merged image was from panels I-K. Scale bar in D applies to A-C and I-L. The scale bar in H applies to E-G.

Figure 21. Individual spiral ganglion neurons demonstrate the classic bipolar and pseudomonopolar shapes**. A**, A bipolar shaped spiral ganglion neuron extended both processes for long distances from the cell body (star) and bifurcate (arrow, arrowhead). The neuron was labeled with anti-β-tubulin antibody. An unpublished image from Mou, K. and Davis, R.L. **B**, A live image of a pseudomonopolar shaped neuron from a Thy-1 YFP preparation. The hair cell orientation was known for this explant culture. The parent axon bifurcation (arrowhead) and the terminal specializations (arrow) indicated on the peripheral side of the process. **C**, The axon length measured from the cell body to the first bifurcation point showed no differences between apical and basal neurons.

Figure 22. Axon outgrowth differences between apical and basal explant cultures. **A-F**, Spiral ganglion neurons were labeled with anti-β-tubulin antibody (red) and anti-synaptophysin antibody (green). **A**, Multiple low magnification images were merged together of an apical neuronal explant. **B-C**, The high magnification images exhibited areas that were typically to apical axonal outgrowth pattern. **D**, Multiple low magnification images merged together of a basal neuronal explant. **E-F**, The high magnification images exhibited areas that were typical to the basal axonal outgrowth pattern. Scale bar in F applies to B-C and E-F

Figure 23. The apical axon outgrowth differences were only exaggerated with the application of neurotrophins. **A-F**, Apical spiral ganglion neurons supplemented with BDNF or NT3 labeled with anti-β-tubulin antibody (red) and anti-synaptophysin antibody (green). A, Multiple low magnification images were merged together of an apical explant supplemented with BDNF. **B-C**, The high magnification images demonstrated apical outgrowth pattern does not change with BDNF treatment. Scale bar in C applies to B. **D**, Multiple low magnification images were merged together of an apical explant supplemented with NT3. **F-E**, The high magnification images demonstrated the apical outgrowth pattern does not change with NT3 treatment. Scale bar in E applies to F.

Figure 24. The basal axon outgrowth differences were only exaggerated with the application of neurotrophins. **A-F**, Basal spiral ganglion neurons supplemented with BDNF or NT3 labeled with anti-β-tubulin antibody (red) and anti-synaptophysin antibody (green). **A**, Multiple low magnification images were merged together of a basal explant supplemented with BDNF. **B-C**, The high magnification images revealed the basal outgrowth pattern does not change with BDNF treatment. Scale bar in C applies to B. **D**, Multiple low magnification images were merged together of a basal explant supplemented with NT3. **E-F**, The high magnification images revealed the basal outgrowth pattern does not change with NT3 treatment. Scale bar in E applies to F.

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Table 1: List of Primary Antibodies

Antibody	Immunogen	Manufacturer	Dilution
Calbindin	Recombinant rat calbindin D-28k	Swant, CB-38a Raised in rabbit Polyclonal antibody	1:100
Calbindin	Chicken calbindin D28k	Swant, 300 Raised in mouse Monoclonal antibody	1:100
class III β-tubulin (TUJ1)	microtubules from the rat brain	Covance, MMS-435P Raised in mouse Monoclonal antibody	1:350
class III β-tubulin	Generated against the same epitope recognized by TUJ1	Covance, PRB-435P Raised in rabbit Polyclonal antibody	1:2,000
HCN 1	peptide(C)KPNSASNSRDDGNSVYPSK, corresponds to the amino acids 6-24 of the rat HCN1 at the intracellular N-terminus	Alomone Labs, APC-056 Raised in rabbit Polyclonal antibody	1:200
MAP-2	Microtubule-associated protein from the rat brain	Millipore, AB5622 Raised in rabbit Polyclonal antibody	1:100
MBP	Human myelin basic protein from brain	Millipore, AB980 Raised in rabbit Polyclonal antibody	1:100
$p75^{NTR}$	Extracellular fragment from the third exon of mouse p75 (amino acids 43-161)	Millipore, AB1554 Raised in rabbit Polyclonal antibody	1:100
$p75^{NTR}$	Cytoplasmic domain of the human p75 neurotrophin receptor	Promega, G3231 Raised in Rabbit Polyclonal antibody	1:400
Sortilin	Mouse myeloma cell line NS0-derived recombinant mouse sortilin	R & D Systems, AF2934 Raised in Goat	1:100
Synaptophysin	rat retina synaptosome Clone SVP-38	Sigma-Aldrich, S5768 Raised in mouse Monoclonal antibody	1:50

Figure 1

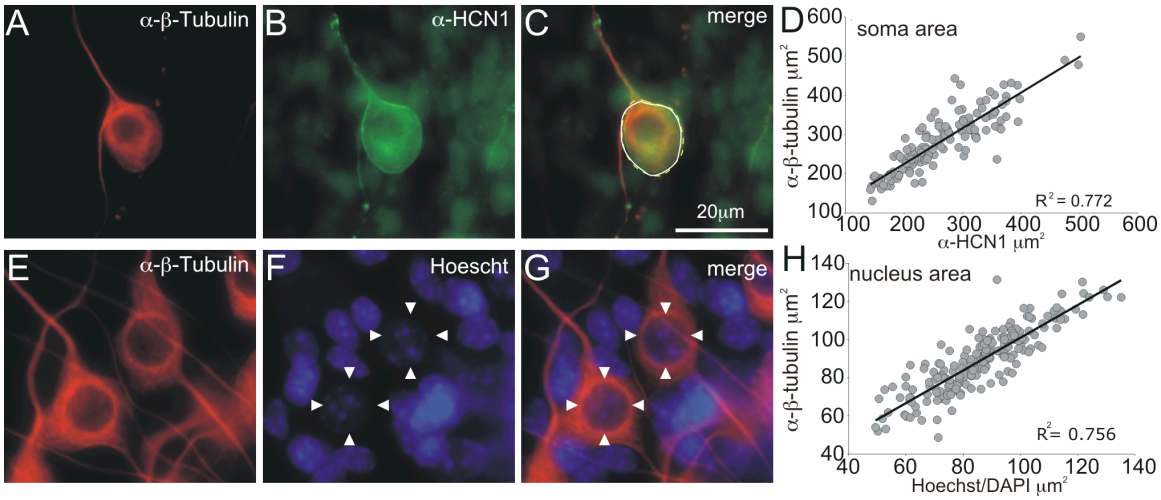

A
α-β-Tubulin
B
α-HCN1
C
merge
20μm
D
soma area
α-β-tubulin μm²
α-HCN1 μm²
R² = 0.772
E
α-β-Tubulin
F
Hoescht
G
merge
H
nucleus area
α-β-tubulin μm²
Hoechst/DAPI μm²
R²= 0.756

Figure 2

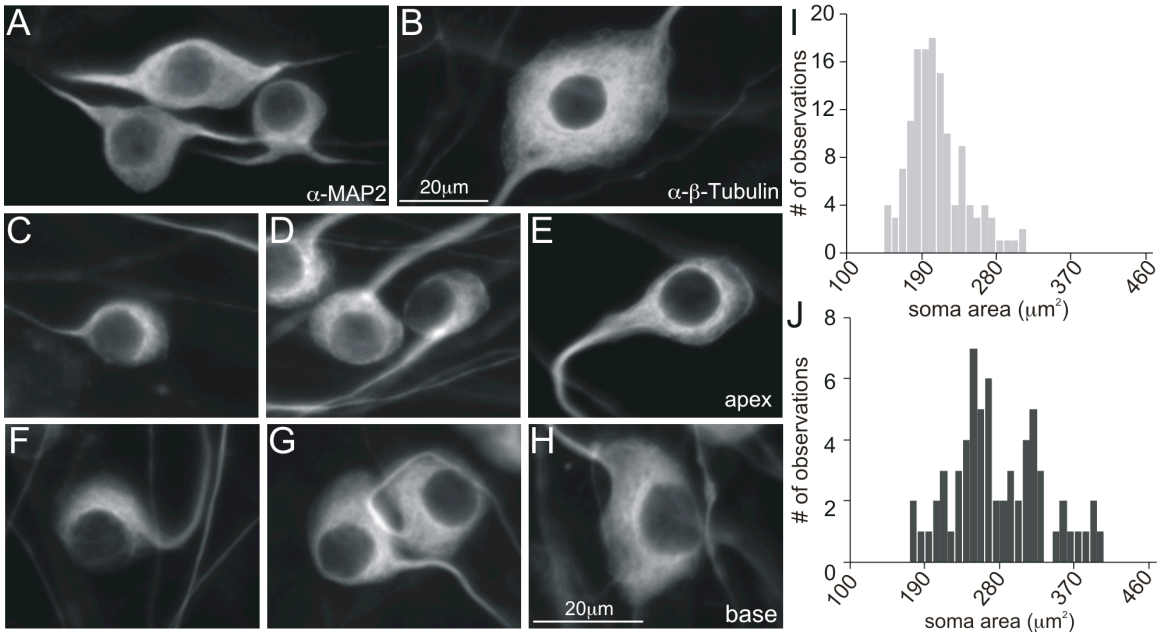

A
α-MAP2
B
20μm
α-β-Tubulin
C
D
E
apex
F
G
H
20μm
base
I
# of observations
soma area (μm²)
J
# of observations
soma area (μm²)

Figure 3

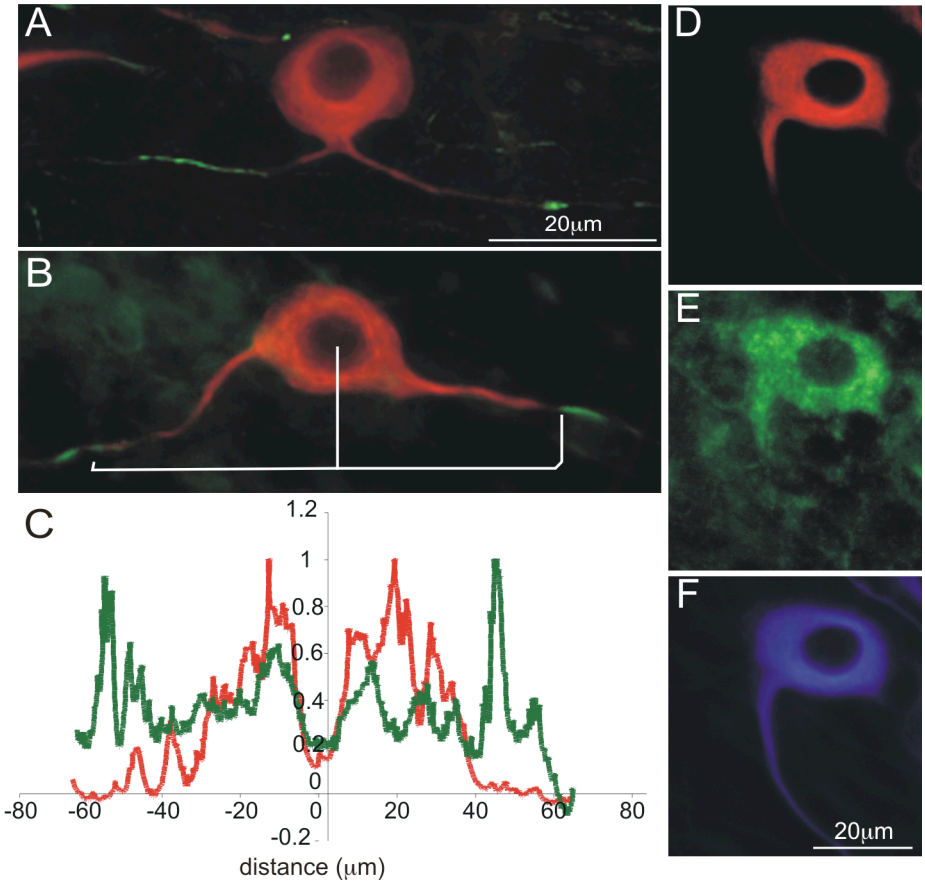

A
20μm
B
C
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-80
-60
-40
-20
0
20
40
60
80
distance (μm)
D
E
F
20μm

Figure 4

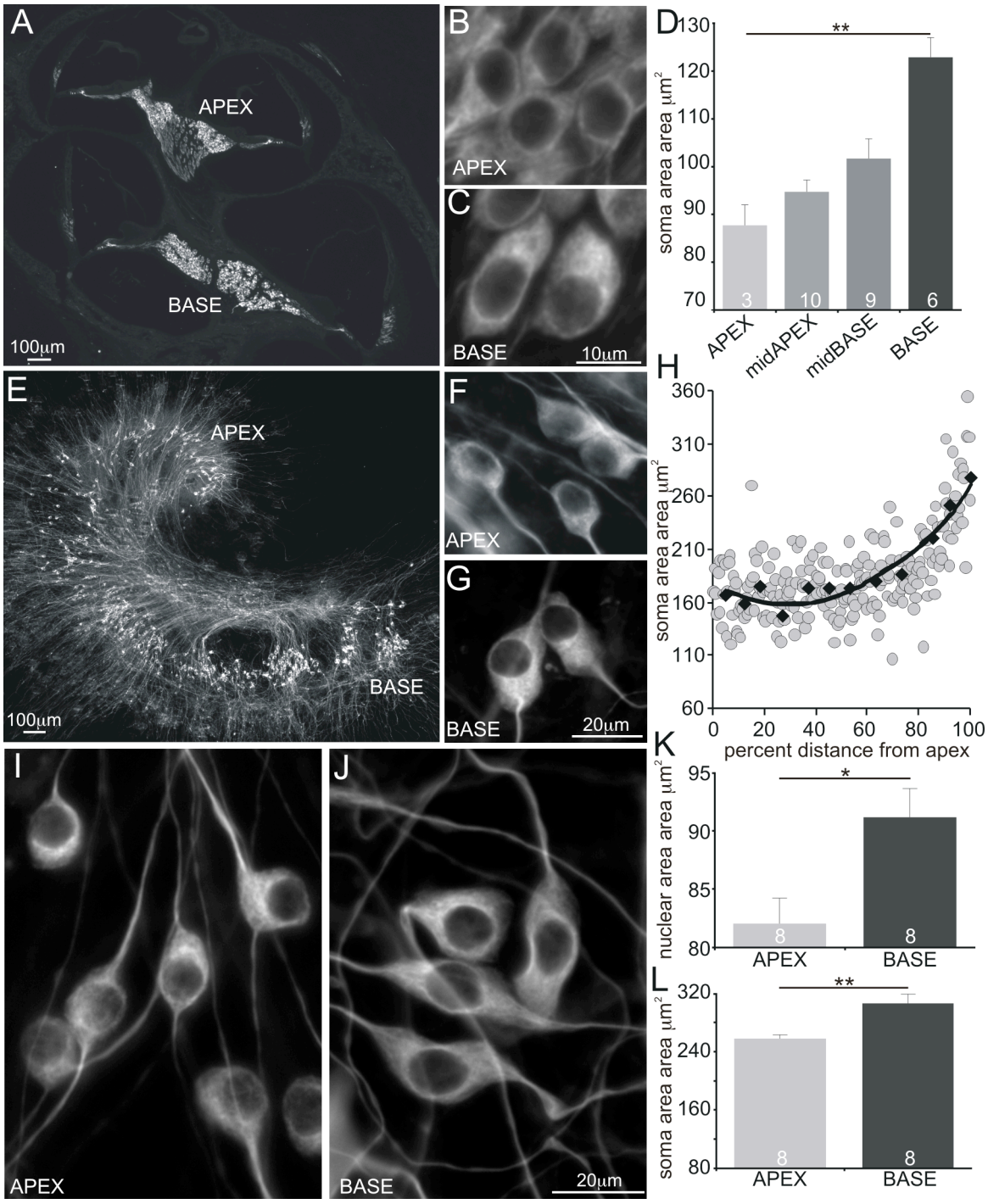

A
APEX
BASE
100μm
B
APEX
C
BASE
10μm
D
**
soma area area μm2
130
120
110
100
90
80
70
3
10
9
6
APEX
midAPEX
midBASE
BASE
E
APEX
BASE
100μm
F
APEX
G
BASE
20μm
H
soma area area μm2
360
310
260
210
160
110
60
0
20
40
60
80
100
percent distance from apex
I
APEX
J
BASE
20μm
K
*
nuclear area area μm2
95
90
85
80
8
8
APEX
BASE
L
**
soma area area μm2
320
240
160
80
8
8
APEX
BASE

Figure 5

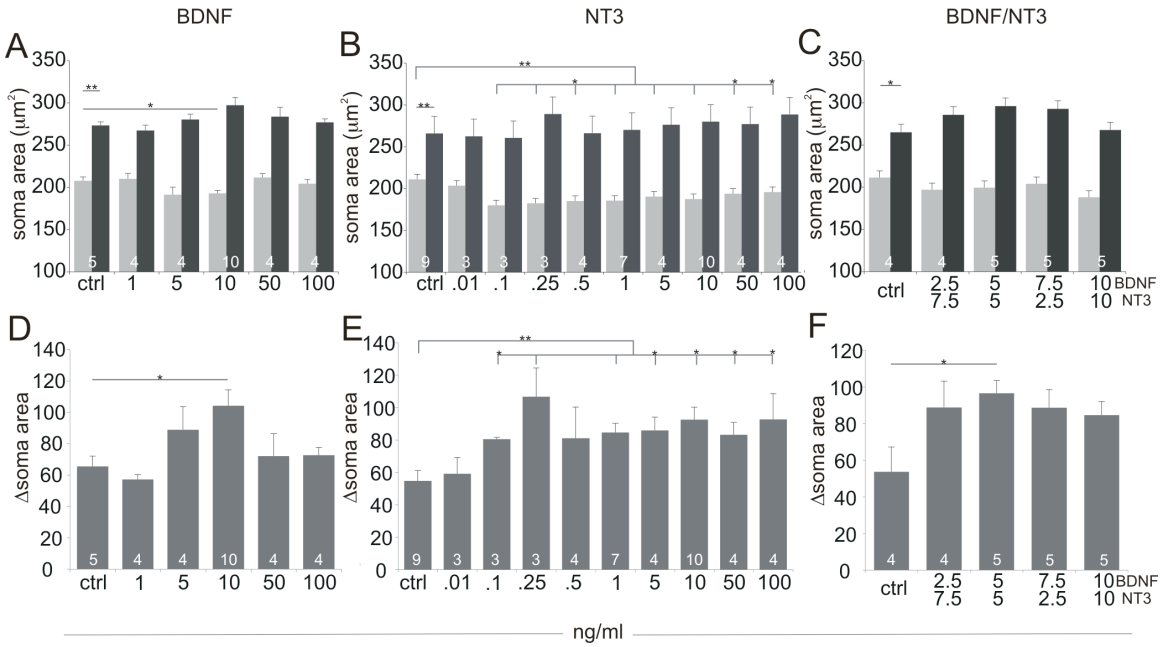
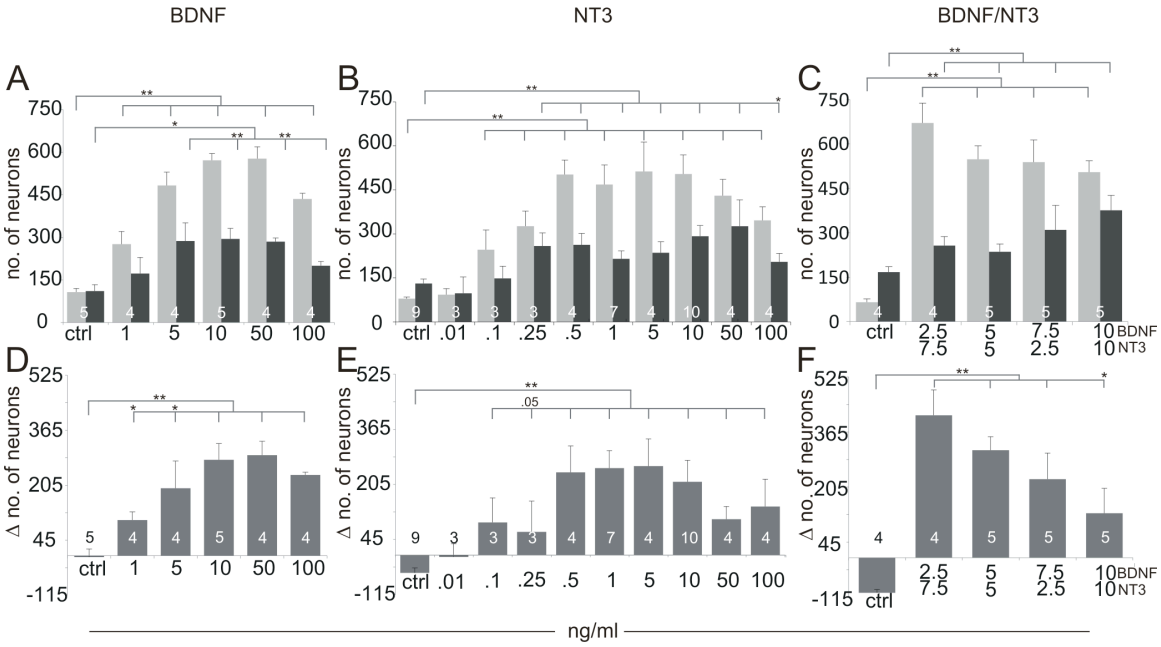

BDNF
NT3
BDNF/NT3
A
B
C
D
E
F
soma area (μm²)
Δsoma area
ctrl
1
5
10
50
100
.01
.1
.25
.5
2.5
7.5
BDNF
NT3
ng/ml

Figure 6


BDNF
NT3
BDNF/NT3
A
B
C
D
E
F
no. of neurons
Δ no. of neurons
ctrl
1
5
10
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100
.01
.1
.25
.5
.05
2.5
7.5
BDNF
NT3
ng/ml

Figure 7

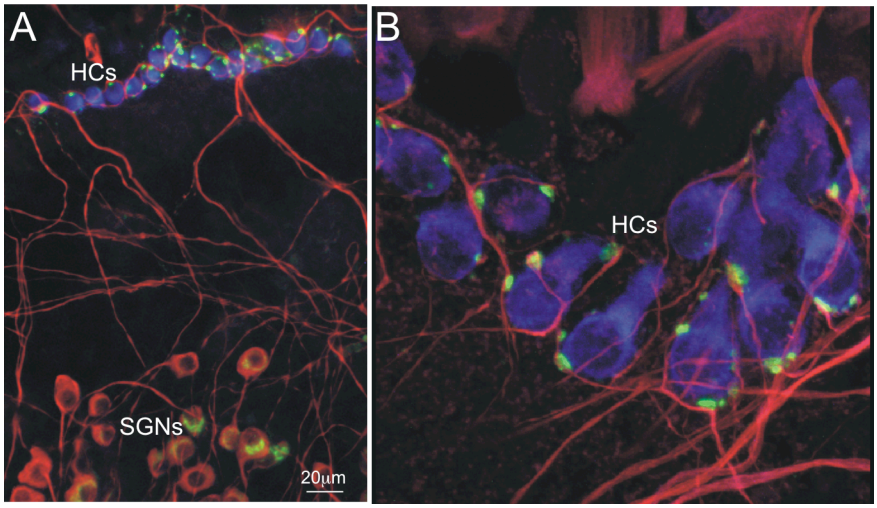


Figure 8

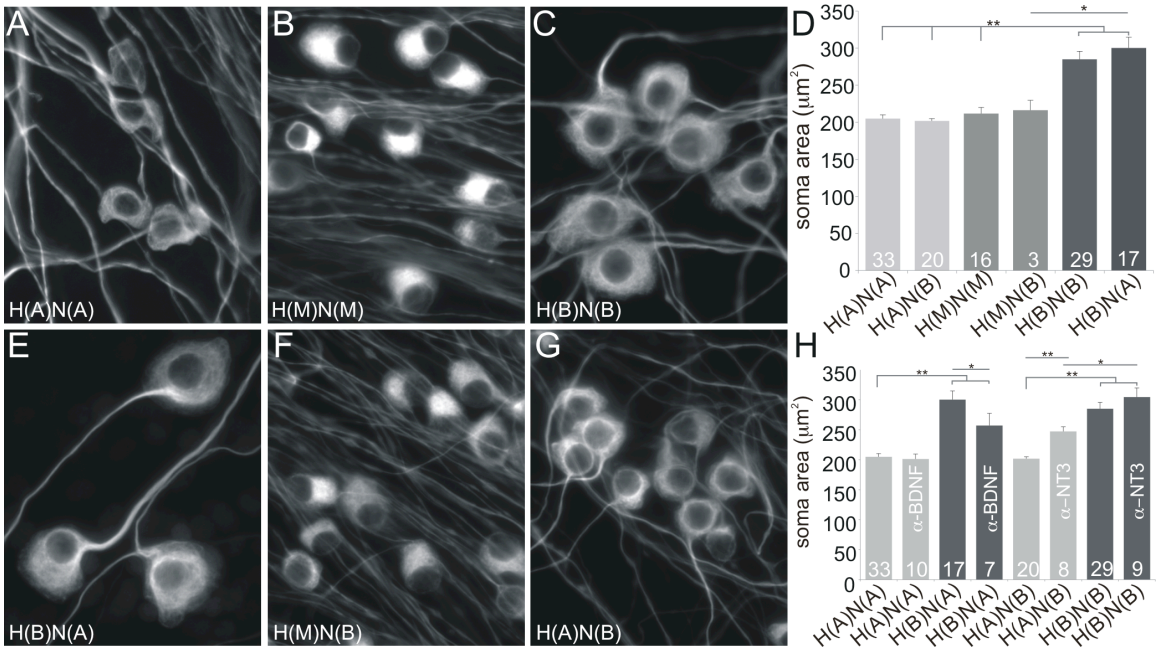

A
H(A)N(A)
B
H(M)N(M)
C
H(B)N(B)
D
soma area (μm²)
350
300
250
200
150
100
50
0
33
20
16
3
29
17
H(A)N(A)
H(A)N(B)
H(M)N(M)
H(M)N(B)
H(B)N(B)
H(B)N(A)
E
H(B)N(A)
F
H(M)N(B)
G
H(A)N(B)
H
soma area (μm²)
α-BDNF
α-NT3
33
10
17
7
20
8
29
9
H(A)N(A)
H(A)N(A)
H(B)N(A)
H(B)N(A)
H(A)N(B)
H(A)N(B)
H(B)N(B)
H(B)N(B)

Figure 9

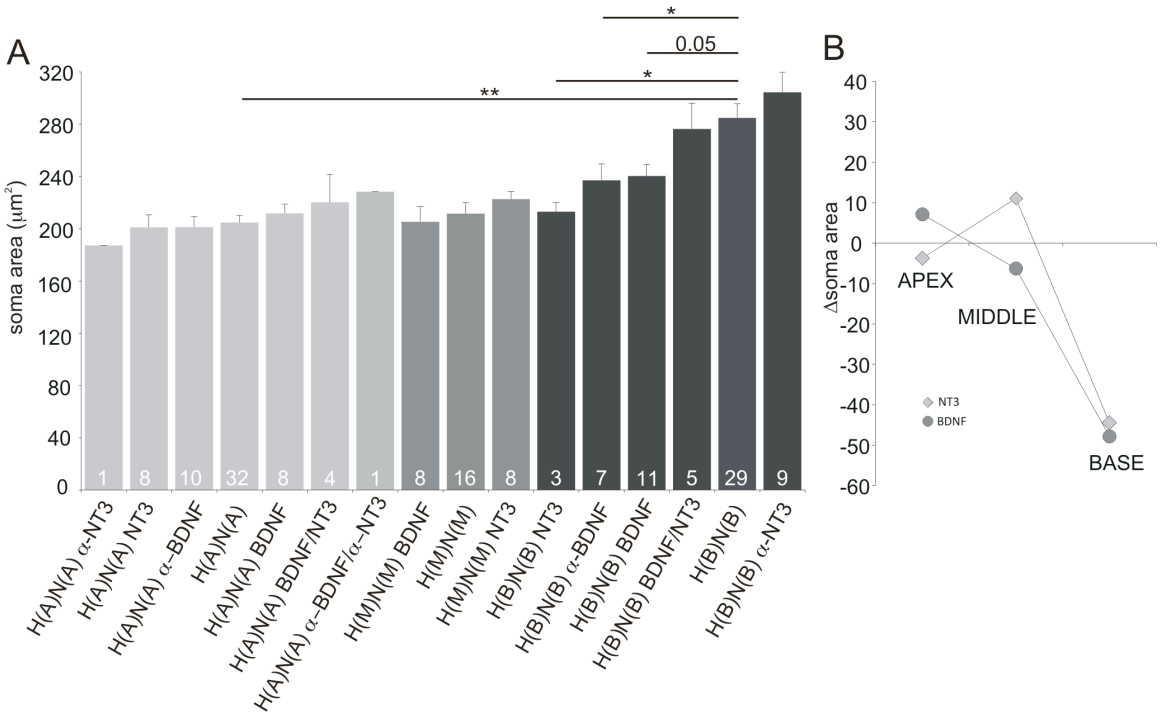

A
B
soma area (μm²)
Δsoma area
*
0.05
*
**
1
8
10
32
8
4
1
8
16
8
3
7
11
5
29
9
H(A)N(A) α-NT3
H(A)N(A) NT3
H(A)N(A) α-BDNF
H(A)N(A)
H(A)N(A) BDNF
H(A)N(A) BDNF/NT3
H(A)N(A) α-BDNF/α-NT3
H(M)N(M) BDNF
H(M)N(M)
H(M)N(M) NT3
H(B)N(B) NT3
H(B)N(B) α-BDNF
H(B)N(B) BDNF
H(B)N(B) BDNF/NT3
H(B)N(B)
H(B)N(B) α-NT3
APEX
MIDDLE
BASE
NT3
BDNF

Figure 10

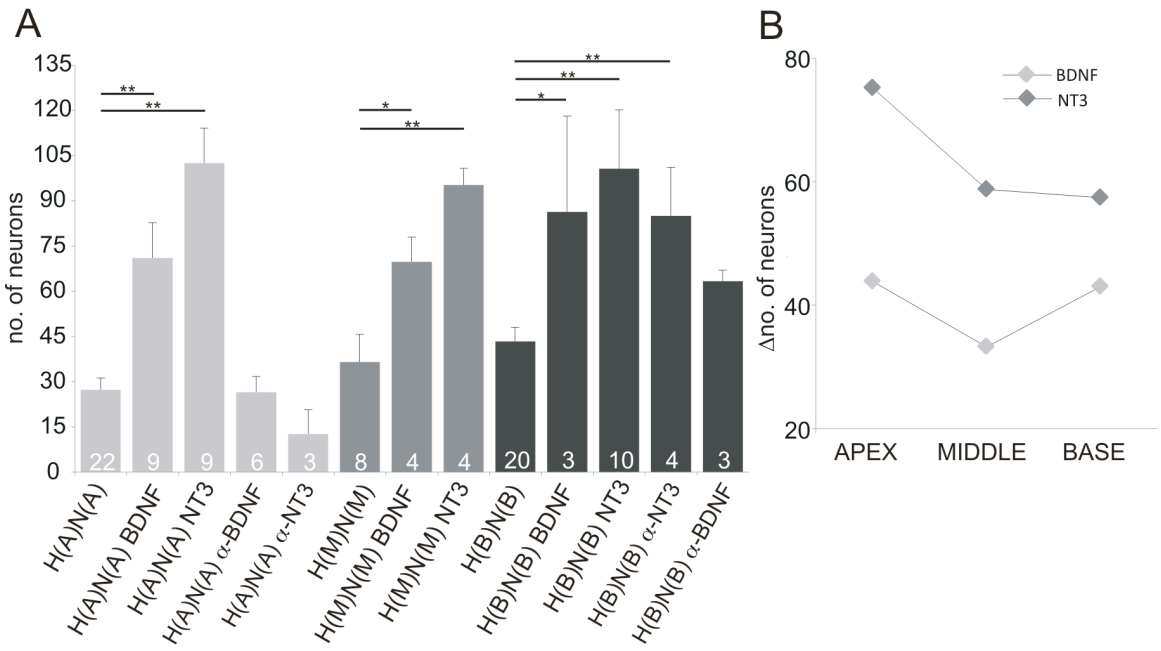

A
no. of neurons
135
120
105
90
75
60
45
30
15
0
22
9
9
6
3
8
4
4
20
3
10
4
3
**
**
*
**
**
**
*
H(A)N(A)
H(A)N(A) BDNF
H(A)N(A) NT3
H(A)N(A) α-BDNF
H(A)N(A) α-NT3
H(M)N(M)
H(M)N(M) BDNF
H(M)N(M) NT3
H(B)N(B)
H(B)N(B) BDNF
H(B)N(B) NT3
H(B)N(B) α-NT3
H(B)N(B) α-BDNF
B
Δno. of neurons
80
60
40
20
BDNF
NT3
APEX
MIDDLE
BASE

Figure 11

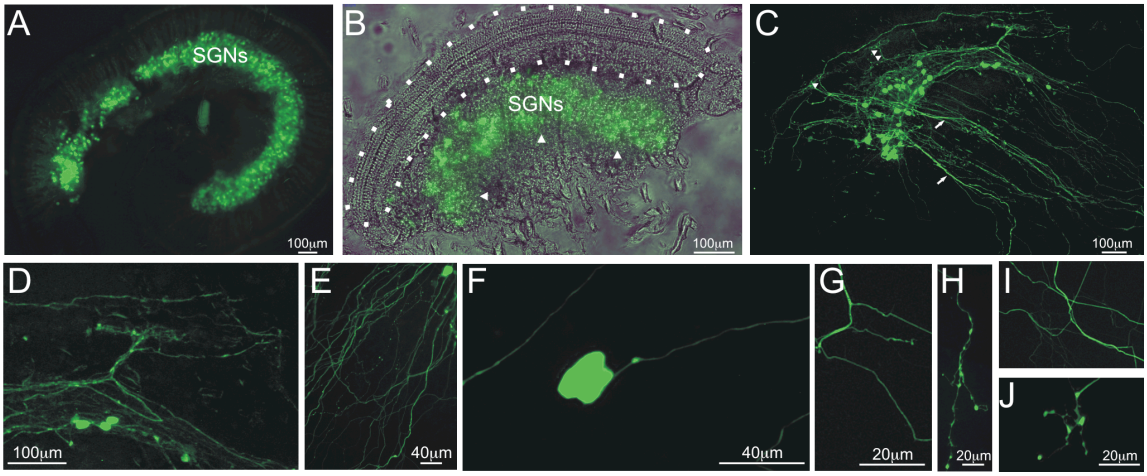

A
SGNs
100μm
B
SGNs
100μm
C
100μm
D
100μm
E
40μm
F
40μm
G
20μm
H
20μm
I
J
20μm

Figure 12

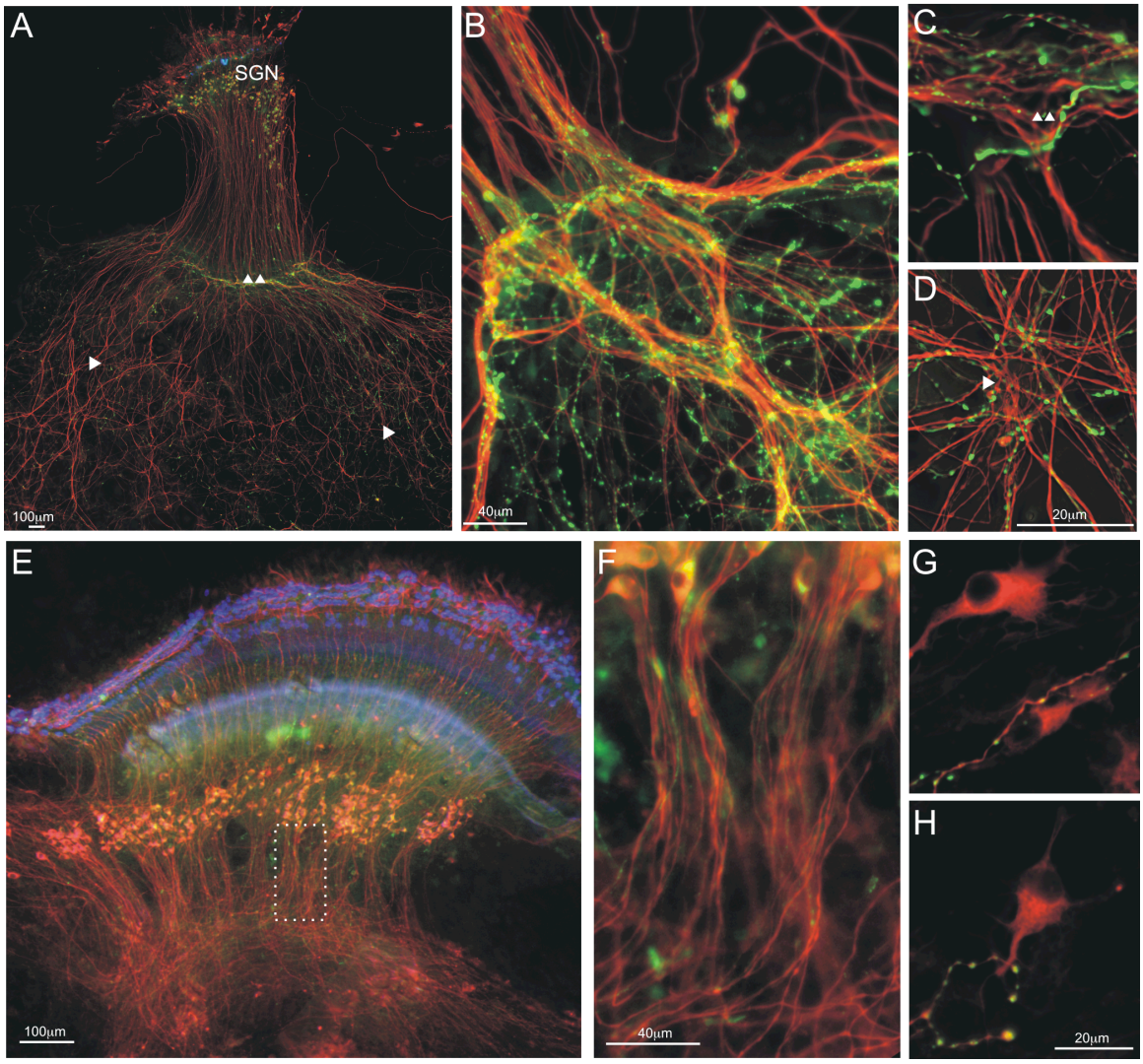


Figure 13

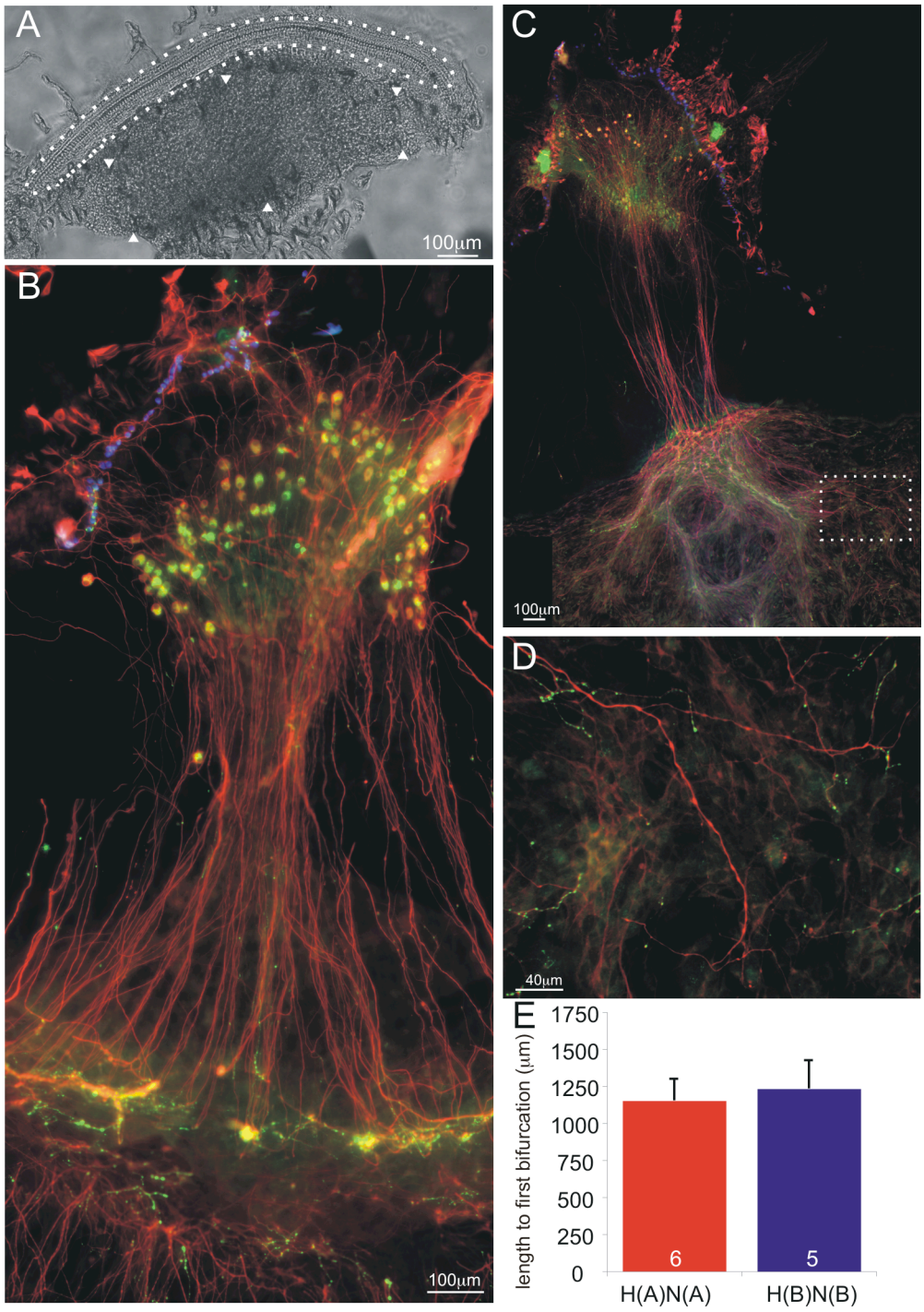

A
100µm
B
100µm
C
100µm
D
40µm
E
length to first bifurcation (µm)
1750
1500
1250
1000
750
500
250
0
6
5
H(A)N(A)
H(B)N(B)

Figure 14

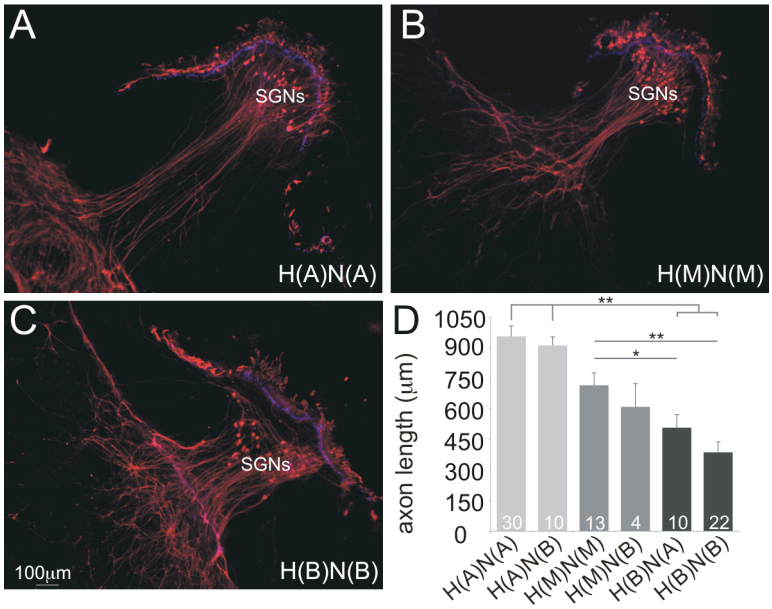

A
SGNs
H(A)N(A)
B
SGNs
H(M)N(M)
C
SGNs
100μm
H(B)N(B)
D
axon length (μm)
1050
900
750
600
450
300
150
0
**
**
*
30
10
13
4
10
22
H(A)N(A)
H(A)N(B)
H(M)N(M)
H(M)N(B)
H(B)N(A)
H(B)N(B)

Figure 15

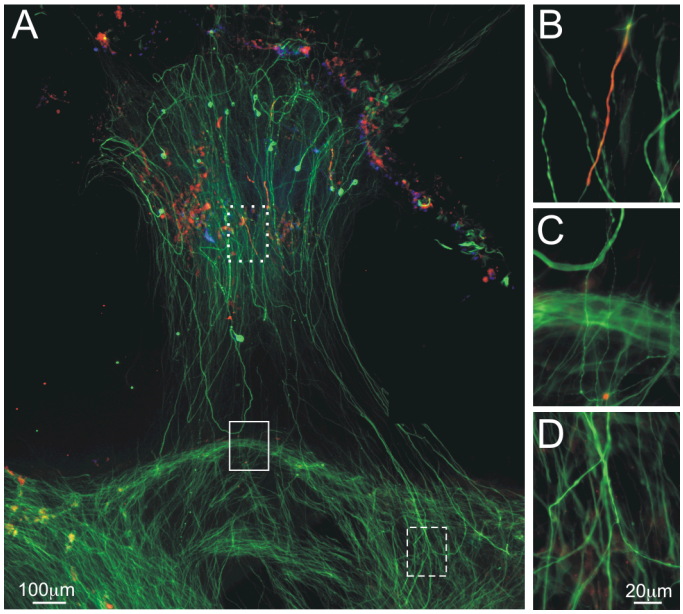


Figure 16

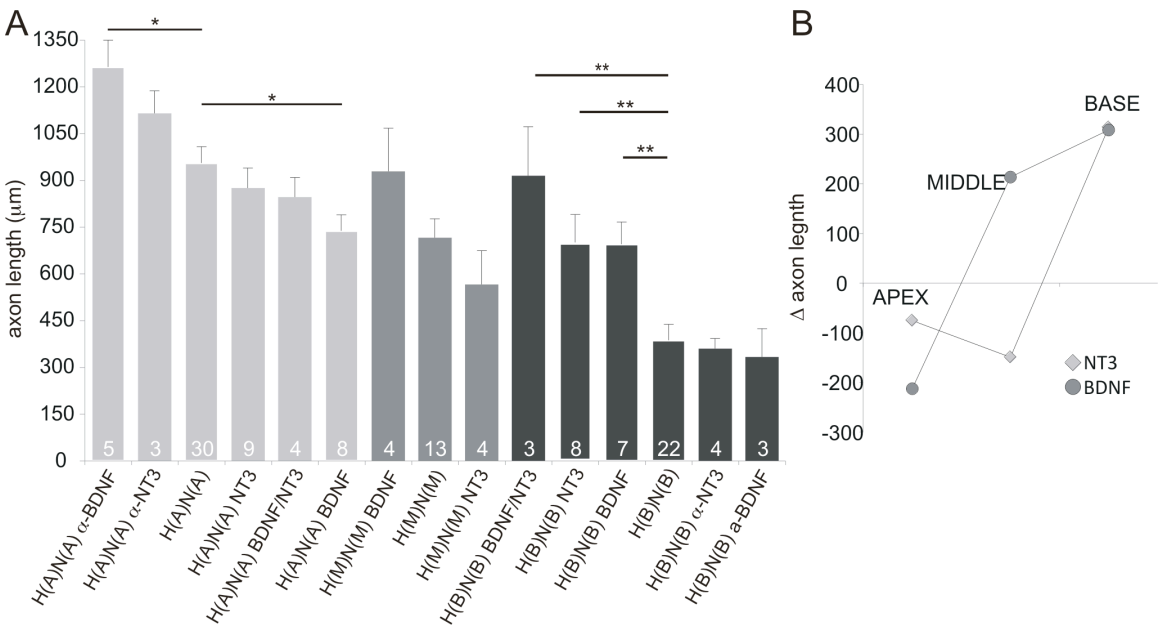

A
B
axon length (μm)
1350
1200
1050
900
750
600
450
300
150
0
5
3
30
9
4
8
4
13
4
3
8
7
22
4
3
*
*
**
**
**
H(A)N(A) α-BDNF
H(A)N(A) α-NT3
H(A)N(A)
H(A)N(A) NT3
H(A)N(A) BDNF/NT3
H(A)N(A) BDNF
H(M)N(M) BDNF
H(M)N(M)
H(M)N(M) NT3
H(B)N(B) BDNF/NT3
H(B)N(B) NT3
H(B)N(B) BDNF
H(B)N(B)
H(B)N(B) α-NT3
H(B)N(B) a-BDNF
Δ axon legnth
400
300
200
100
0
-100
-200
-300
BASE
MIDDLE
APEX
NT3
BDNF

Figure 17

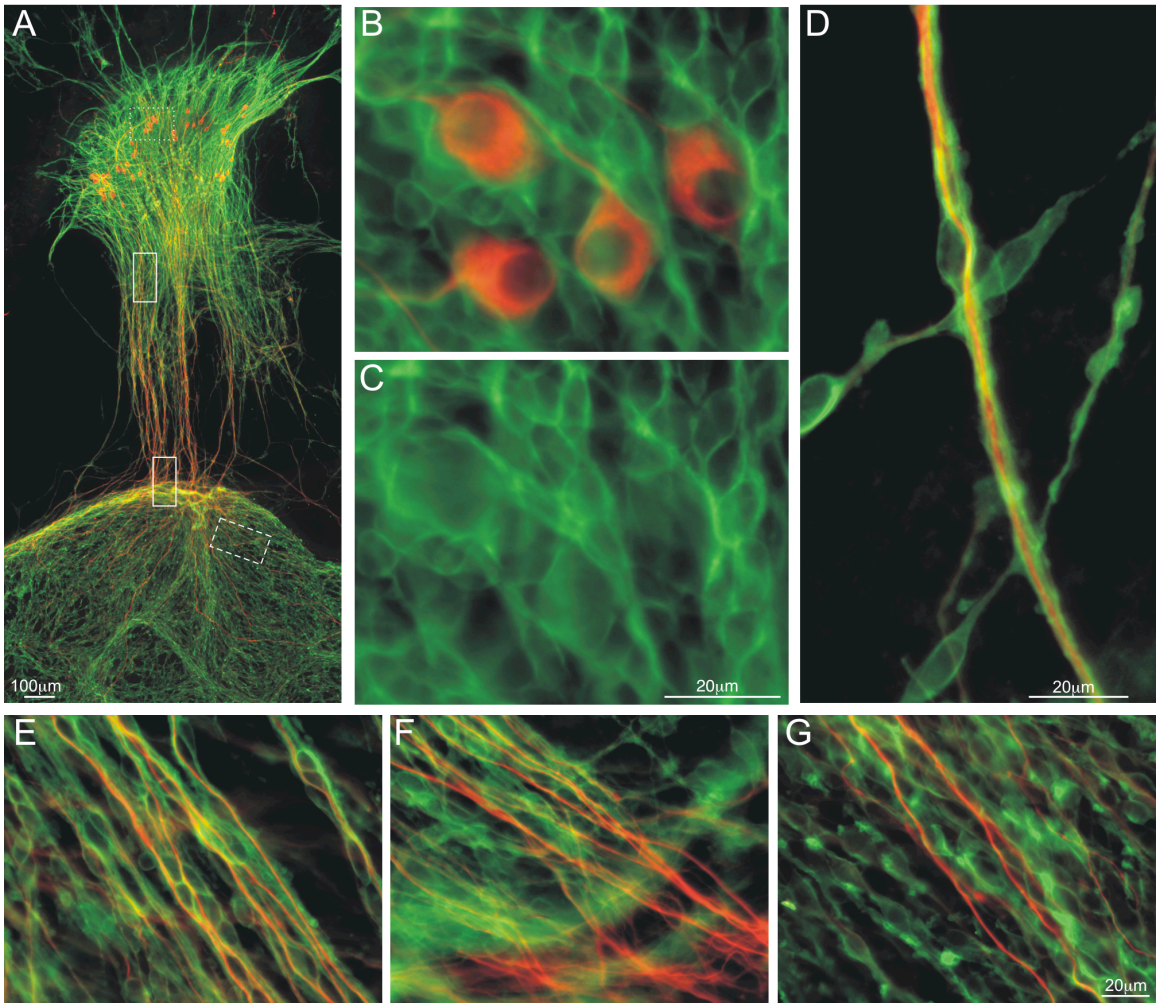


Figure 18

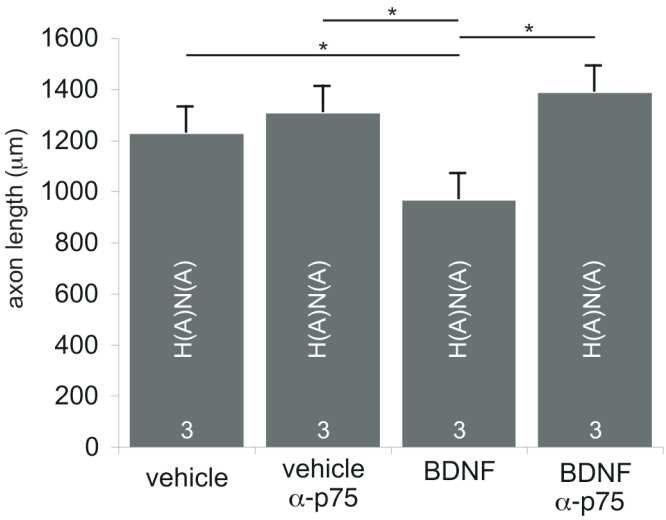

*
*
*
1600
1400
1200
1000
800
600
400
200
0
axon length (μm)
H(A)N(A)
H(A)N(A)
H(A)N(A)
H(A)N(A)
3
3
3
3
vehicle
vehicle
α-p75
BDNF
BDNF
α-p75

Figure 19

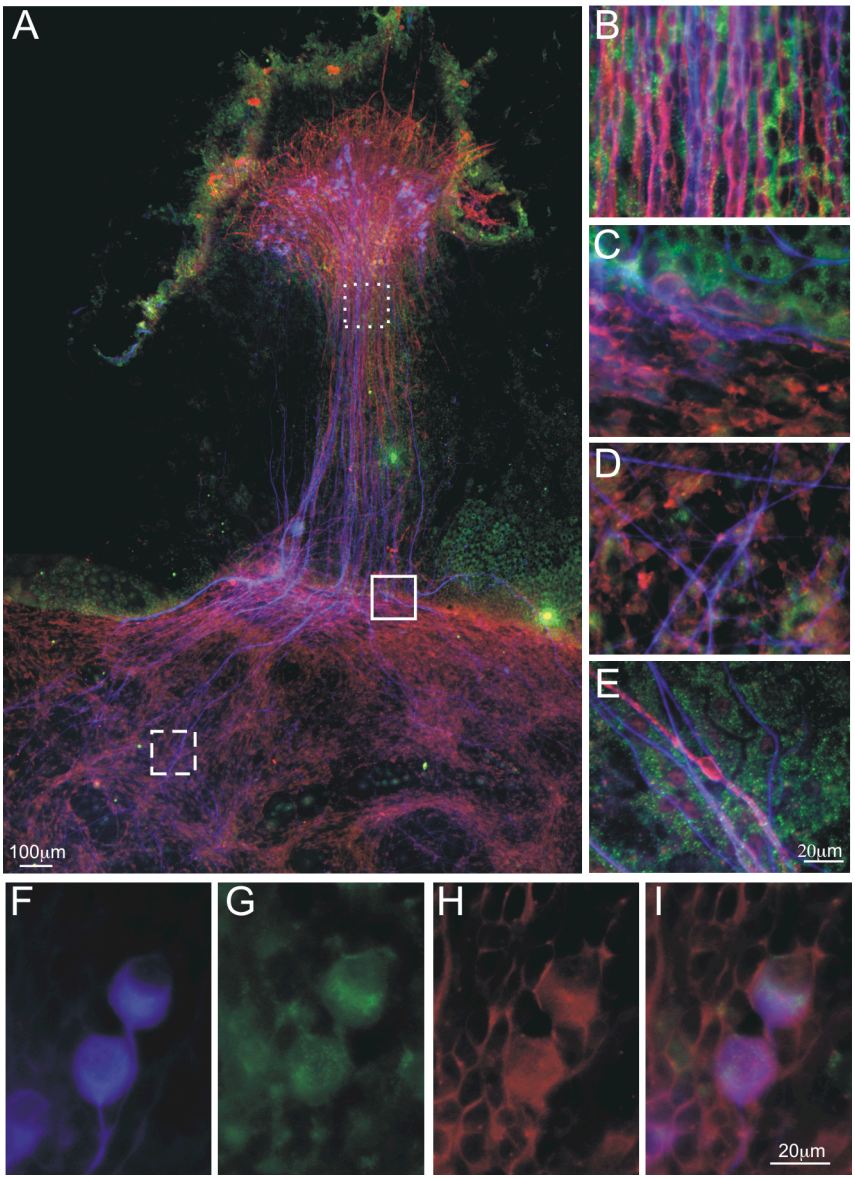


Figure 20

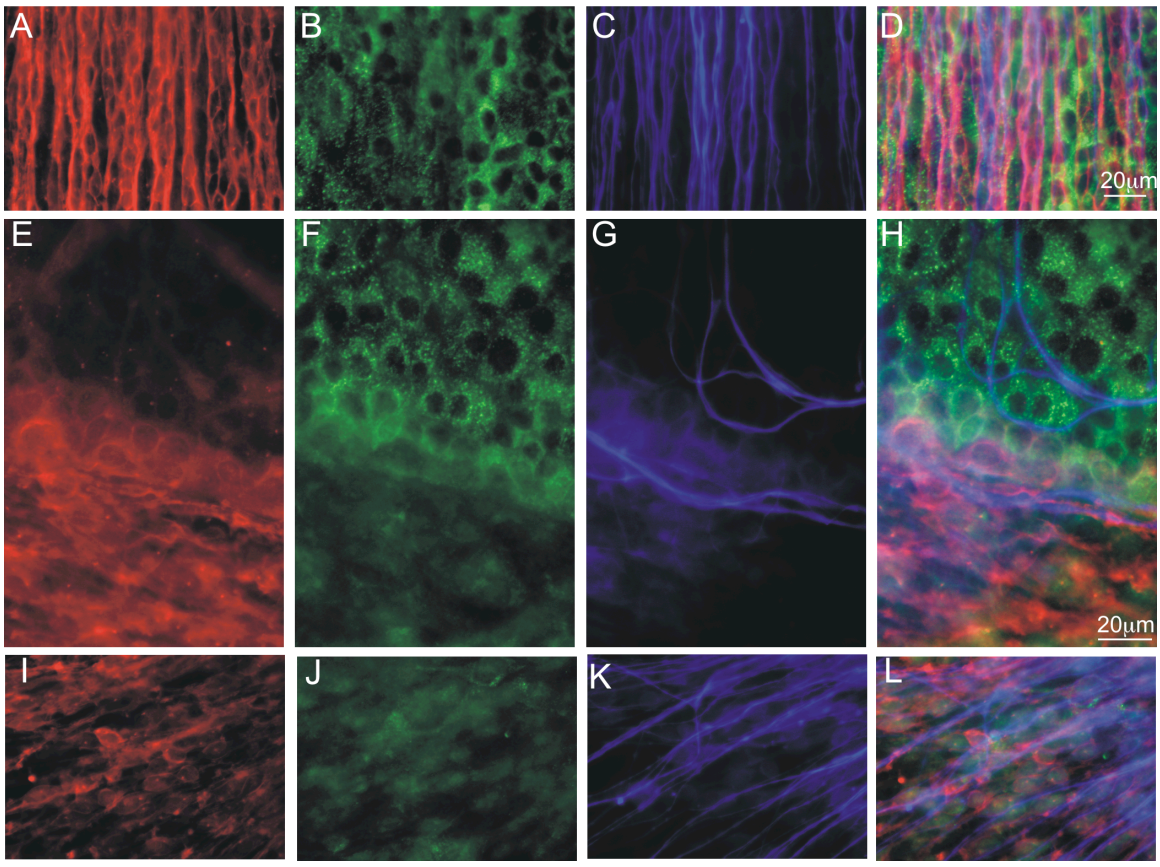


Figure 21

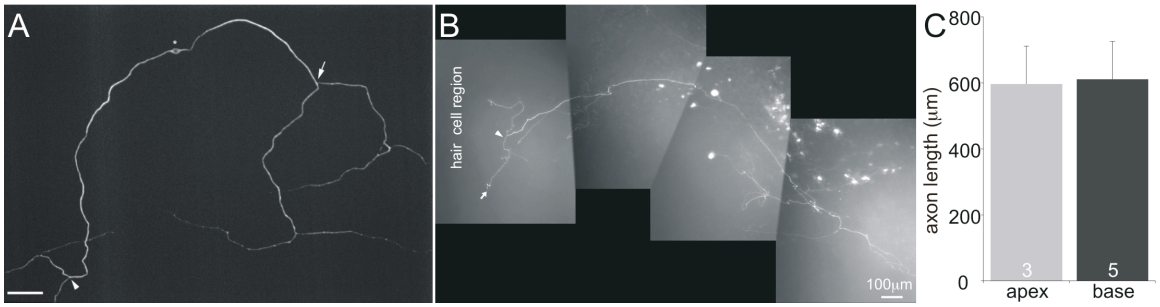

A
B
hair cell region
100µm
C
800
600
400
200
0
axon length (µm)
3
5
apex
base

Figure 22

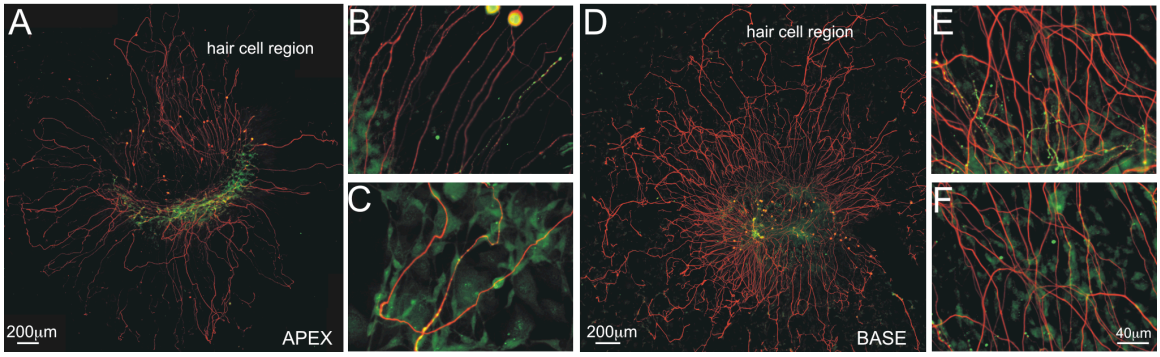

A
hair cell region
200µm
APEX
B
C
D
hair cell region
200µm
BASE
E
F
40µm

Figure 23

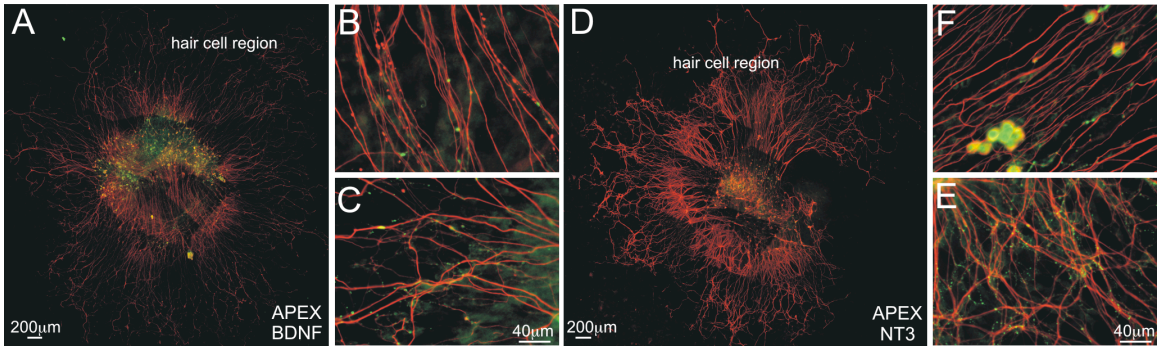
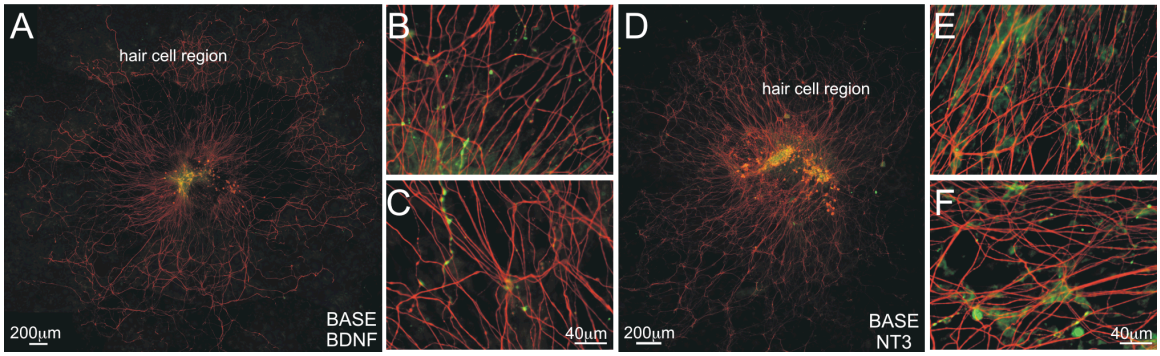

A
hair cell region
200μm
APEX
BDNF
B
C
40μm
D
hair cell region
200μm
APEX
NT3
F
E
40μm

Figure 24


A
hair cell region
200μm
BASE
BDNF
B
C
40μm
D
hair cell region
200μm
BASE
NT3
E
F
40μm