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Protein Stripe Concentration Gradient Effects on Type 1 vs. Type 2 Spiral Ganglion Neurite Termination and Turning Behavior

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Andysheh Kamgar-Parsi

Committee in charge:

Professor Allen F. Ryan, Chair
Professor Yimin Zou, Co-Chair
Professor Andrew Chisholm

2013
The Thesis of Andysheh Kamgar-Parsi is approved and it is acceptable in quality and form for publication on microfilm or electronically:

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University of California, San Diego

2013
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ABSTRACT OF THE THESIS

Protein Stripe Concentration Gradient Effects on Type 1 vs. Type 2 Spiral Ganglion Neurite Termination and Turning Behavior

by

Andysheh Kamgar-Parsi

Master of Science in Biology

University of California, San Diego, 2013

Professor Allen F. Ryan, Chair
Professor Yimin Zou, Co-Chair

The study aimed to evaluate semaphorin and laminin protein gradients role on type 1 and type 2 SGN guidance. We created striped depletion protein gradients of semaphorin (Sema3a and Sema5a) and ECM proteins (Laminin), and looked at their effects on type I and type II SG neurite termination and turning in vitro. Little is known about guidance cue effects on type II SGNs path to OHC targets in the Organ of Corti, and whether guidance cue concentration gradients play a role in differential turning between type I and type II SGNs. Sema3a, Sema5a and laminin are expressed in the
sensory epithelium and play respective roles growth cone and axonal guidance; therefore, are good guidance cues candidates. Guidance and concentration gradient properties of these proteins were determined by SG neurite projection quantification. Explants were cultured on plates with alternating protein stripes of Sema3a, Sema5a, and laminin, respectively, versus PLL alternating stripes. Type I SG neurite projections avoided Sema3a, Sema5a, and laminin stripes, while type IIs, which were also repulsed by Sema5a and laminin, had neurite projections land on Sema3a ON stripes. Sema3a possibly has chemoattractant effects on type II SG neurite projections. Type 1 SG neurites favored higher concentrations of laminin at 5 µg/mL, while all other protein stripes depletion gradients had no significant effect on either type I or type II SGNs. These proteins possibly serve as guidance cues for both type I and type II SGN neurons, however whether the gradient concentration guides neurite turning behavior isn’t clear.
I. INTRODUCTION

1. Hair Cells and Spiral Ganglion Neurons

The conversion of sound from physical waves to neural signals occurs in the sensory epithelium of the cochlea, the receptor organ for hearing. The cochlear sensory epithelium is known as the organ of Corti, and sits upon the basilar membrane. This membrane distributes the frequencies of sound energy to different regions along the length of the cochlea, which runs from the base (high frequencies) to the apex (low frequencies). The organ of Corti contains sensory cells known as hair cells that transduce the physical vibrations of sound waves into neural signals that are subsequently sent to the central nervous system. There are two main types of hair cells: inner hair cells (IHCs) and outer hair cells (OHCs). The hair cells are arranged along the apex-base axis of the organ of Corti, where there are one row of IHCs and three rows of OHCs, separated by supporting cells called pillar cells (Rubel and Fritzsch, 2002). Both IHCs and OHCs contain stereocilia, which are cellular projections that are deflected when sound waves delivered to the cochlea cause the underlying membrane to vibrate. The motion of the stereocilia opens transducer channels that depolarize the hair cells and activate afferent synapses at the base of the cell. These in turn depolarize primary auditory neurons known as spiral ganglion neurons (SGNs), which conduct impulses to the central nervous system. The SGNs are located in the bony modiolus at the center of the cochlea, where a coiled channel, the Rosenthal’s canal, holds the somata of the SGNs. Each cell body extends a peripheral dendrite that innervates the organ of Corti, and a central axon that projects via the auditory nerve to the central nervous system (Nayagam et al., 2011).
2. SGN Type 1 vs. Type 2: Innervations, Morphology, and Function

Within the spiral ganglion, there are two distinct populations of bipolar SGNs. These populations are completely segregated in their afferent hair cell target innervations and eventually are pruned to eliminate inappropriate connections that are made during mammalian auditory development (Echteler, 1992). There is a group of neurons that make up 90-95% of these neurons that are classified as type I SGN. The dendrites of ten to twenty of these type I SGNs converge onto each IHC (Huang et al., 2012; Rubel and Fritzsch, 2002). IHCs and type I SGNs are primarily responsible for encoding sound stimuli. The other group of neurons, and perhaps the more enigmatic, make up only about 5% of SGNs, and are classified as type II SGNs. Yet, despite being only 5% of the SGN population, type IIs innervate exclusively all three rows of OHCs, as well as some of the pillar cells (Jagger and Housley, 2003; Fechner et al., 2001, Spoendlin, 1973, Kim, 1984). Each dendrite from the type II SGN branches and contacts many OHCs (Barclay et al., 2011).

Type I SGNs have been well described morphologically as having thicker radial dendrites and axons, and having a larger soma area in comparison to type II’s, which have thinner fibers, and a smaller soma area (Brown et al., 1987; Berglund and Ryugo 1987). Type I dendrites typically appear as single, unbranched, and myelinated fibers, whereas type IIs are unmyelinated and branch out in *en passant fasion* on the OHCs (Barclay et al., 2011). Type I SGNs have been well characterized via electrophysiology, and their physiology closely resembles the responses of many neurons in the central auditory system. Type II’s have not been studied to the same extent, partially due to their small, unmyelinated nature (Reid et al., 2004). Thus the function of type II SGNs
remains undetermined, but they are thought to play a role in the active 'cochlear amplifier' associated with a motor function of the OHCs (Ashmore and Kolston 1994; Liberman et al., 2002). This motor function is mediated by an inter-membrane “molecular motor” known as prestin, a voltage-sensitive molecule that alters the length of the OHC in response to potential change. The amplification of sound by the OHCs increases the sensitivity of the cochlea by 100-fold, and is responsible for the exquisite frequency selectivity of the cochlea (Oghalai, 2004). A study by Jagger and Housley (2003), looked at the in vitro electrophysiology of SGN type II’s between P7-P10 and found there is a distinct firing pattern that was described as having: A-type current, higher AMPA receptor firing, and lower spatial resolution than type I SGNs (Housley and Jagger, 2003). The few recordings that have been made in vivo indicate that, like the type I neurons they are highly tuned, but they have higher thresholds than type I SGNs (Roberston, 1984).

3. SGN Neuronal Projections

The type I SGN dendrite travels a straight path from the spiral ganglion to the organ of Corti, and synapses onto a single IHC. The axon of each type II neuron projects to the large central cells of the cochlear nucleus, and are organized tonotopically such that the frequency organization of the cochlea (base-to-apex) is preserved in the central auditory system.

   Type IIIs project much differently. Like type I dendrites, type II neurites pass through the osseous spiral lamina directly to the organ of Corti; however, instead of innervating the IHCs, they pass underneath them and project across the organ to the OHC region. From there, they turn towards the cochlear base and project for about 500-600
micrometers, ascending up within the organ until they reach the bases of the OHCs. As mentioned earlier, one dendrite of each type II innervates onto many OHCs (Barclay et al., 2011). Type II SGNs also project in a frequency-specific distribution to the cochlear nucleus (Berglund and Brown, 1994), but they project to granule cell regions on the edges of the nucleus (Brown et al., 1987).

4. SGN Innervation Throughout Development

Although, adult type Is are exclusive for IHCs and type IIs are exclusive for OHCs and support cells, there is some relative plasticity during development. This plasticity is short-lived and is replaced by specific, refined, and separated innervations of SGN type Is and IIs prior to functional onset. One study by Echteler et al. (1992) showed that at birth, Gerbil SGN neurites contacted both IHCs and OHCs; however, by P6, they had separated and the type I and type II SGNs had differentiated and synapsed onto their respective hair cell targets.

In a study by Barclay et al., (2011), it was found that SGN type IIs in the mouse experience extensive apoptosis after birth, from P0-P2, and this may be linked to synaptic pruning and refinement of the type II’s relatively abundant early afferent dendritic connections to OHCs. An additional study in mouse by Huang et al. (2007), found a similar pattern of hair cell innervation, with limited segregation of type Is and type IIs to IHCs and OHCs prior or up to birth; however from P3-P6, there is extensive neuronal remodeling in the organ of Corti that leads to type I and type II segregation to their respective hair cell innervations.
Unlike humans, which have a mature auditory system between 22-25 weeks of gestation (Pundir et al., 2012), rodent cochlear development is refined and matured postnatally, which makes them excellent animal model systems. However, as mentioned earlier, the window of opportunity for assessing at type II SGNs in mouse cochlea is limited due to the neuronal cell death that severely limits their numbers within the first few postnatal days. While it is clear that the type I and type II neurons take very different paths during development, the molecular basis for this is unknown. It seems likely that specific guidance factors are involved, and that the responses of the two SGN types are different for different factors.

5. NF vs. Peripherin Staining

Neurofilament (NF), a major component in the neuronal cytoskeleton that functions in maturing and maintaining nerve fibers, is a common marker used to immuno-stain for SGN explant projections (e.g. Romand et al., 1990). However, since NF is present both in type I and type II SGNs, it is difficult if not impossible to differentiate between the two types of SGNs without resorting to observation of ultrastructural or electrophysiological differences. One of the more effective ways of differentiating between type I and type II SGNs is through peripherin staining. Peripherin is a type III intermediate filament (IF) that has been found throughout the peripheral and central nervous system. In the cochlea, its function remains unclear. However, peripherin has been found preferentially in type II and not in type I (Lallemand et al., 2007; Barclay et al., 2011). The study by Lallemand et al., (2007) showed that staining for peripherin was seen before birth within all rat cochlear neurons; however, from P0-P3, peripherin
expression was gradually restricted to the type II neuronal population and their projections. Barclay et al. (2011) found that peripherin preferentially labels mouse type II neurons from birth on. Type II SGN neural outgrowth is effected by a peripherin knockout model, therefore suggesting the necessity for peripherin for type II SGN survival and dendritic projection (Barclay et al., 2011).

Hafidi, (1998), found that within the spiral ganglion of the adult rat cochlea that there was positive reactivity for type II SGNs, while type I neurons were negative. He also found that in the organ of Corti, there were peripherin-positive fibers that formed bundles beneath the OHCs, and sent branches that ended as boutons contacting the OHCs. A study done on human fetal cochlea at 27 weeks of gestation, reported that peripherin immunoreactivity was specifically distributed in some small SGN central and peripheral extensions, especially in fibers that contacted the lower part of the OHCs (Despres et al., 1994).

6. BDNF and NT3 – Spiral Ganglion Neuron Survival Factors

Type II spiral ganglion neurons require brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), both part of the neurotrophin family, for neurite outgrowth. These two factors are expressed in the inner ear and have been demonstrated to promote neurite outgrowth in various studies in both rat and mouse inner ear (Ernfors et al., 1992; Pirvola et al., 1992; Ylikoski et al., 1993; Aletsee et al., 2001). Barclay et al., (2011) found that BDNF plays a critical role in type II spiral ganglion neuron survival; this is consistent with the decrease of BDNF postnatally in mice, which is temporally associated with the large amount of type II SGN apoptosis mentioned above. NT-3 has been shown
to support the survival of type I SGNs in vitro (Kondo et al., 2013). Deletion of the BDNF gene results primarily in the loss of vestibular neurons and type II neurons, while, on the other hand, deletion of NT-3 results in a more than 80% reduction of SGNs (Ernfors et al., 1995; Bianchi et al., 1996). Knockouts for trk C, the high affinity NT-3 receptor, also results in loss of SGNs, especially at the base of the cochlea (Fritzsch et al., 1998). This is consistent with the loss of type I SGNs. However, those SGNs that do survive, despite loss of BDNF or NT-3, are still able to reach their targets via dendritic projections (Fritzsch et al., 1997, 1999), suggesting normal responses to guidance factors.

7. Guidance Cues and Guidance Cue Gradients

Guidance cues have long been known to affect the projection and synaptogenesis of developing nerve fibers. The projection of neurites is controlled by many guidance factors that influence the rate and direction of their projections. These cues can function at short and long ranges, and can influence the formation of axon bundles or fasicles, (Kolodkin and Tessier-Lavigne, 2011). Short range cues that affect neuronal projections typically function as contact attraction or repulsion cues. Long range cues are soluble and act via chemotraction or chemorepulsion. In a developing nervous system, these guidance cues are often found at areas called critical points or choice points, and these molecular “landmarks” can guide axons and dendrites to their immediate and final targets. Using invitro tissue culture assay is one potential way to assess whether a protein contains attractive or repellent properties, and thus can play a role as a guidance cue. In addition to the presence or absence of guidance cues, gradients have been shown to
provide additional directional guidance. Gradients have the property of being able to
guide neurites in a specific direction, either up or down a concentration difference.

8. Protein Depletion Gradients

In order to study the potential for guidance proteins to influence SGN type I and
type II fiber growth, turning, and termination, we have applied the proteins onto a cell
culture surface using a microfluidic system. A microfluidic system controls fluid
distribution on a micro-scale (Folch et. al, 2008) and has been used in various protein
biology experiments (Maerkl, 2011; Chung et. al 2005; Lin et al. 2005). Fluids can
contain molecular compounds. There are many recent developments in microfluidic
technology that have increased the precision and accuracy of molecular fluid
administration in a cellular environment and subsequent quantification of said molecular
compounds. The advantage of microfluidic systems is that the area in which molecular
compounds are administered can be controlled and modified within the molecular sensing
range of neurites.

In the case of this experiment, the specific microfluidic system used to distribute
Sema3a, Sema5a, and Laminin produced a depletion gradient. This assay, developed by
Hatch et al., (2001), and modified by Fosser and Nuzzo (2001), uses microfluidic
channels opened at both ends, and incorporates a reservoir of a specific concentration of
protein or proteins of interest at one end of the channel. Diffusion along the length of the
channel creates a gradient, and binding to the substrate transfers this gradient to the
growth substrate. This results in a series of parallel gradient stripes, upon which neuronal
explants can be cultured. This is indicated in figure 4 (Folch and Keenan, 2007), where
the long rectangular shapes represent the channels. Brighter areas represent higher molecular concentrations, while less bright areas represent lower molecular concentration. A concentration gradient is similarly represented by the orange triangular gradient model from high to low concentration seen on the left of figure 5. The protein concentration along the stripes cannot be exactly determined since the binding of protein to the substrate can only be estimated. However, it can be estimated and semi-quantitated using standards.

9. Semaphorin 3A and Semaphorin 5A as Guidance Cues

Sema3a and Sema5a are members of the semaphorin family and have been characterized as extracellular cues, usually found in nervous development systems as repulsive cues (Luo et al. 1993). However, other studies have shown semaphorins to be chemo-attractants as well (de Castro et al., 1999; Bagnard et al., 2001; Hilario and Rodino-Klapac, 2009). Semaphorins can act either as long or short range guidance cues. Sema3a was originally called collapsin-1 due to chick NGF-responsive growth cone collapse and neurotrophin modulation in response to Sema3a (Tuttle and O’Leary, 1998). Sema3a is a diffusible axonal guidance molecule that can act in many functional ways, including: being a chemorepellent for axonal outgrowth, maintaining growth cone collapse in mice hippocampal cells and Xenopus retinal cells. Sema3a is also a chemo-attractant for dendrite growth, modulating elaboration of second and third order dendritic branches in pyramidal neurons and dendritic orientation of the cerebral cortex, and has additional axonal pathfinding functions in the PNS and CNS (Schlomann et al., 2009; Campbell et al., 2001; Fenstermaker et al., 2004; Gu et al., 2003; Polleux et al., 1998;
Polleux et al., 2000; Sasaki et al., 2002). Sema3a has also been found to play important roles in chick embryo dorsal root ganglion cells and helps regulate growth cone motility among other functions (Dontchev and Letourneau, 2003). A study by Song et al., (1998), found that Xenophus neuronal growth cone repulsive responses to Sema3a can be changed from repulsion to attraction by pharmacologically activating the cGMP signaling pathway. Sema3A binds to the neuropilin-1 (nrp1) receptor (He and Tessier-Lavigne, 1997). While nrp1 binds the Sema3a ligands, an intracellular response is not generated unless neuropilin1 forms a receptor complex with its coreceptor, plexin-A1 (Webber and Raz, 2006). Sema3a has also been found to interact with the other class 3 semaphorin protein receptors, such as Plexin A2, Plexin A3, and nrp-2 (Guan et al., 2006).

Sema5a is a transmembrane semaphorin, containing seven thrombospondin type-1 repeats, and is linked to axon guidance functions in different parts of the CNS and PNS. Sema5a is known to be a bifunctional axon guidance cue for mammalian midbrain neurons that can either chemically signal neurons to migrate towards or away from a Sema5a protein gradient (Hilario et. al., 2009). A study, by Kantor et. al., (2004), showed that the thrombospondin type-1 repeats of Sema5a can physically interact with chondroitin sulfate proteoglycans (CSPGs) and heparin sulfate proteoglycans (HSPGs), and be converted to an inhibitory or attractive guidance cue, respectively, for developing axons in the fasciculus retroflexus, a tract of diencephalon fibers connected with limbic function. Plexin-B3 was found by Artigiani et al., (2004), to be a high-affinity receptor specific for Sema5A, where Sema5a mediates functional responses in plexin-B3-expressing cells (either fibroblasts, epithelial and primary endothelial cells).
10. Semaphorin 3A and Semaphorin 5A Expression in Ear

Semaphorin 3A (Sema3a) and Semaphorin 5A (Sema5a) are found to be expressed in different areas of the ear during embryonic development. Evaluation of data from Genepaint, a well-established database of genetic expression patterns in mouse embryos, shows Sema3a to be expressed in the spiral ganglion and in the sensory epithelium, just lateral to the hair cell area (see appendices, figure 1a) (Sema3a, E15.5 C57BL/6 Mouse Cochlea, Genepaint). Sema5a, on the other hand, is expressed just below the sensory epithelium, but not in the spiral ganglion (see appendices, figure 1b) (Sema5a, E14.5 C57BL/6 Mouse Cochlea, Genepaint). The expression outside of the ganglion is most important for guiding neurites.

Nrp-1, Sema3a’s functional co-receptor, has been found to be expressed in the inner ear (see appendices, figure 3a), and Nrp-1 knockouts have aberrant effects on vestibular ganglion afferent axonal pathfinding (Gu et al., 2003). Plexin-A1 has been found to be expressed in the ear as well, specifically in the spiral ganglion, as well as its afferent and efferent cochlear fibers (Murakami et al., 2001). Plexin-B3 mRNA has been found to be expressed in the inner ear of zebrafish, with 78.72% identity to Plexin-B3 receptor found in mus musculus (Coimbra et al., 2002) and some expression in sensory epithelium (see appendices, figure 3c).

11. Extracellular Matrix Molecules

Extracellular matrix proteins have been known to be involved with axonal guidance. One example of an ECM involved with axonal guidance is laminin. Laminin is classified as a glycoprotein heterotrimer that is assembled from alpha, beta, and gamma chain subunits, secreted and placed into cell-associated extra-cellular matrices to help
scaffolding of many basement membranes. Laminins can ‘self-assemble’, bind to other surrounding matrix macromolecules, and have an impact on shared cell interactions that is regulated by integrins, dystroglycan, and other receptors (Colognato and Yurchenco, 2000). Different domains of laminin are related to different parts of laminin function including neurite guidance and cell adhesion, where during embryonic development, laminin is arranged in the CNS either as cell-surface-associated (CSA) deposits or placed in big polymeric aggregates randomly within the ECM space (Luckenbill-Edds, 1997). The laminin peptide sequence IKVAV (Ile-Lys-Val-Ala-Val) in the E8 domain has been found to play a role with neurite outgrowth, cell attachment, and other cell related behavior (Tashiro, 1989). The laminin isoform LN-1 (the same isoform used in this study) was isolated from Engelbreth-Holm-Swarm mouse sarcoma cells (Timpl and Rohde, 1979).

12. Laminin expression in the Ear

Laminin has been found to be expressed in both mammalian and avian cochleas, located close to SGN afferent tracts that coincide temporally with organ of Corti innervation. Laminin-1 isoform (LN) is expressed along the SGN tracts towards the developing organ of Corti, and can enhance neurite outgrowth in vitro (Aletsee et al., 2001). Laminin staining is present beneath IHCs and is seen in the mouse spiral ganglion from P0, where it increases at P2 and eventually tapers off and decreases at P14 (Cosgrove and Rodgers, 1997). In the fetal human cochlea at 11 and 15 weeks there was strong signal of laminin present in the basement membrane of the inner ear (Yamashita et al., 1991). In the adult guinea pig, there was laminin staining in areas surrounding the SG
cells and neurites (Takahashi and Hokunan, 1992). Laminin staining has also been seen around the chick basal papilla, an avian structure that is very similar to the mammalian organ of Corti, and it lies directly within the cochlear ganglion fiber tracts (Hedmond and Morest, 1991). These different animal model systems show the close relationship between laminin expression and cochlear neuronal development.

13. Laminin as a Guidance Cue

Laminin is known as a permissive growth cue in cell culture mediums, which allows outgrowth of neurites rather than growth cone collapse, like Sema3a (Bonner and O’Conner, 2001; Lander et al., 1985; Hall et al., 1987). When laminin is present, in protein stripes, laminin has helped to direct different types of neurite growth (Bahr and Wizenmann, 1996; Clark et al., 1993; Turney and Bridgmann, 2005). Spiral ganglion neurites have been shown to track laminin stripes and preferentially terminate onto higher concentrations of laminin, while avoiding relatively lower concentrations of laminin (Evans et al., 2007). Therefore, concentration gradient of laminin may play a role in the turning and termination of SGNs when presented in a depletion gradient.

14. SGN response to Laminin

Laminin is known to influence spiral ganglion neurites in vitro. As mentioned earlier, laminin is expressed in the spiral ganglion tracts leading to the organ of Corti, and it inflicts a dose-dependent increase in number and length of spiral ganglion neurites in vitro (Aletsee et al., 2001). There is a possibility that the increase of SGN length is mediated by mitogen-activated protein kinase (MAPK) Erk and G protein Ras. It was
found that the MAPK (MEK) inhibitor U0126 was able to decrease neurite length and number even with laminin present, suggesting that the neurite length is mediated through the MAPK (MEK) signaling pathway (Aletsee et al., 2001). However, K-Ras signaling appears to only affect MEK-dependent neurite length increases, but not affect MEK-dependent neurite number changes, pointing to the fact that this neuronal parameter is most likely mediated by a different intracellular pathway (Aletsee et al., 2001). There is also the possibility of the involvement of rho GTPases, such as rho, rac, and cdc42, that help regulate SGN outgrowth (Brors et al., 2003).
II. MATERIALS AND METHODS

1) Preparing Tissue Culture Plates

Stripe molds were generated by applying silicone rubber (Slygard 184, Silicone elastomer, Dow Corning Inc.) to a template with vertical 100 μm wide stripes and 100 μm apart produced by photolithography. The silicone matrix was cured for 72 hours at room temperature under a vacuum before being cut into 4 x 4 mm pieces. The silicone molds were then brushed clean with cotton swab in 70% ethanol, sterilized with 100% ethanol, and air dried in a sterile hood overnight. The silicone molds were placed groove side down in the center of a 24 well culture plate (costar, Corning Inc.), with the grooves placed horizontal to base of the plate. Sterile phosphate buffer saline (PBS) was added to the left side of the mold and was drawn into the channels by suction applied at the right side edge of the mold. The right side edge was then sealed with human recombinant human recombinant Sema3a (R&D Systems), human recombinant Sema5a (R&D Systems), or human recombinant laminin (Sigma-Aldrich) protein concentration (5 μg/mL and 2 μg/mL), as to prevent evaporation of fluid from channels during overnight incubation at 37 °C. This will also allow time for the protein to fill in the grooved wells and create the protein depletion gradient. Recombinant Bovine Serum Albumin or BSA (Sigma-Aldrich) will be used as a control due to the fact that it isn't a known guidance cue, and thus should not have any significant effect on ON or OFF protein stripe termination or SG neurite turning behavior.

After incubation, the silicone molds were removed in the hood, and the protein channels were then washed twice with PBS. Each well was then filled with 250uL of 5ug/ml PLL solution (Sigma-Aldrich) and incubated at 37 °C for 1 hour. After 1 hour
incubation, wells were washed with PBS three times, and then were filled with 170 µL of primary attachment medium: Dulbecco’s modified eagle medium (DMEM) (Gibco), 10% fetal calf serum (Sigma-Aldrich), 25mM HEPES buffer (Gibco) and 300 µg/mL penicillin (Sigma Aldrich).

2. Spiral Ganglion Dissection

Neonatal (P0.5) C57/6BL mice were deeply anesthetized before decapitation. The skull was opened midsagitally, the brain was removed and the temporal bones extracted. The cochlear capsule was opened and the stria vascularis as well as the Organ of Corti were removed. The spiral ganglion was then dissected from the modiolus and transferred immediately into primary cell culture medium after being excised. SG tissue was cut into five-eight equal pieces separating the apical, middle and basal turns of the spiral ganglion before being placed in primary attachment medium.

3. Spiral Ganglion Tissue Culture

After dissection, cell culture plate was incubated overnight at 37 °C in primary attachment medium for 24 hours. After 24 hours, the culture medium was replaced with serum-free maintenance media (DMEM (Gibco), 25mM HEPES buffer (Gibco), 6mg/ml glucose (Gibco), 300ug/ml penicillin (Sigma-Aldrich), and 30ug/ml N2-supplement (Gibco). Maintenance medium was also supplemented with 100ug/ml of recombinant BDNF (R&D Systems) and 100 µg/mL of recombinant NT-3 (millipore) to support type II SG neurite outgrowth. Explants were cultured in a humidified incubator with 5% CO2 and 37 °C for 72 hours.
4. Immunofluorescent Staining

After 72 hour incubation in serum-free maintenance medium, the medium was removed, and the plate was washed twice with PBS. The SGN explants were then fixed for 20 min at room temperature with 4% paraformaldehyde (Affymetrix), then washed twice with PBS (Gibco). The fixed explants were permeabilized for 10 minutes with 5% Triton X-100 (Sigma-Aldrich). After permeabilization, the wells were washed twice with PBS, and then both SG neurites and protein gradient stripes were labeled with the appropriate antibody. SGNs were labeled using a rabbit monoclonal antibody against neurofilament-200 (1:500; Sigma-Aldrich). Type II SG neurites were labeled using a polyclonal chicken antibody against the 57 kDa intermediate filament peripherin (1:500; Thermo Scientific). Sema3a was labeled with polyclonal rabbit antibody (1:100; abcam). Sema5a was labeled with sheep antibody (1:100; R&D systems). Bovine Albumin Serum (BSA), control, was labeled with polyclonal rabbit antibodies (1:500; Life Technologies). Each primary antibody was labeled separated, for the respective protein stripe gradients, Neurofilament-200 staining, and Type II Peripherin staining, and each incubated in parafilm overnight 4 degrees C. In between each respective primary antibody incubation, wells were washed twice with PBS. After primary antibody incubations, wells were washed twice with PBS, before they were incubation with secondary antibody for 2.5 hours with light protection (foil wrapped) at room temperature. Triple immuno-staining was done, were samples were incubated with fluorescein isothiocyanate (FITC) (1:100; Jackson Immunoresearch), Alexa-fluor 594 (1:100; Jackson Immunoresearch), and Dylight-405 (1:00; Jackson Immunoresearch) conjugated secondary antibodies against rabbit, sheep, and chicken primary antibodies respectively. After 2.5 hour incubation,
plates were washed twice with PBS to get rid of excess secondary to make for clearer IF images.

5. Data Analysis

At least 6 explants were evaluated for each concentration of Sema3a, Sema5a, and Laminin protein gradient stripes. Digital images were obtained on an fluorescence microscope (Olympus IX70). Images were stitched together and optimized using Adobe Photoshop (Adobe Systems Inc.). The neurite terminations and gradient concentration preferences were marked and counted to determine substrate preference and any preferential turning. Statview 5.0 was used to perform ANOVA and Fischer tests to determine statistical significance.
III. RESULTS

1. Type 1 vs. Type 2 SG neurite terminations on Sema3a ON Stripe versus PLL OFF Stripe

Type I SG neurites cultured on plates with alternating stripes of Sema3a versus PLL tended to have significantly more neurites ending on PLL OFF stripes than on Sema3a ON stripes. This occurred for both concentrations of Sema3a (5 µg/ml and 2 µg/ml) (Fisher's PLSD Test: 5: p = 0.0039*, 2: p = 0.0005*). There was significantly less type 2 SG neurite OFF stripe termination when compared to type 1 SG neurite OFF stripe termination for both concentrations of Sema3a (Fisher's PLSD Test: 5: p = 0.0062**, 2: p < 0.0001***). Type 2 SG neurites projections preferred Sema3a ON Stripes rather than PLL OFF stripes (Fisher's PLSD Test: 5: p = 0.0643, 2: p = 0.0226*). Refer to figure 1 for the Sema3a 5 µg/ml conditions and figure 2 for the Sema3a 2 µg/ml in the appendix.

2. Type 1 vs. Type 2 SG neurite terminations on Sema5a ON Stripe versus PLL OFF Stripe

Type 1 SG neurites cultured on plates with alternating stripes of Sema5a versus PLL tended to have significantly more neurites ending on the PLL OFF stripes than on Sema3a ON stripes. This occurred for both concentrations of Sema5a (5 µg/ml and 2 µg/ml) (Fisher's PLSD Test: 5: p = 0.0393*, 2: p < 0.0001*). Type II SG neurites exhibited the same response at both concentrations (5 µg/ml and 2 µg/ml), where significantly more of type II SG neurites had their projections terminate on the PLL OFF stripes rather than the Sema5a ON stripes (Fisher's PLSD Test: 5: p = 0.0393*, 2: p =
0.0001*). Refer to figure 3 for the Sema5a 5 µg/ml conditions and figure 4 for the Sema5a 2 µg/ml in the appendix.

3. Type 1 vs. Type 2 SG neurite terminations on LN ON Stripe versus PLL OFF Stripe

Type 1 SG neurites cultured on plates with alternating stripes of LN versus PLL tended to have significantly more neurites ending on the PLL OFF stripes than on LN ON stripes. This occurred for both concentrations of LN (5 µg/ml and 2 µg/ml) (Fisher's PLSD Test: 5: p = 0.0148*, 2: p<0.0001*). Type II SG neurites exhibited a similar response at both concentrations (5 µg/ml and 2 µg/ml), where more of type II SG neurites had their projections terminate on the PLL OFF stripes rather than the LN ON stripes (Fisher's PLSD Test: 5: p < 0.1611, 2: p < 0.0280*). Refer to figure 5 for the LN 5 µg/ml conditions and figure 6 for the LN 2 µg/ml in the appendix.

4. Type 1 vs. Type 2 SG neurite terminations on BSA (Control) ON Stripe versus PLL OFF Stripe

Type 1 SG neurites cultured on plates with alternating stripes of BSA versus PLL did not have any experience any significant effect by BSA ON stripes and projected their termination on as many BSA ON stripes as they did PLL OFF stripes at both concentrations (Fisher's PLSD Test: 5: p = 0.5502, 2: p = 0.7694). Type II SG neurites did not experience any significant effect by BSA either at both concentrations (5 µg/ml and 2 µg/ml), and also had their neurite projections land on as many ON BSA stripes as PLL
OFF stripes (Fisher's PLSD Test: 5: p = 0.4739, 2: p = 0.7694). Refer to figure 7 for the BSA 5 µg/ml conditions and figure 8 for the BSA 2 µg/ml in the appendix.

5. Type 1 vs. Type 2 SG neurite preferential turning to High Sema3a Protein Gradient Concentration vs. Low Sema3a Protein Gradient Concentration vs. No Sema3a Protein Gradient Concentration Turning Preference.

Type 1 SG neurites cultured on plates with protein depletion gradient of Sema3a did not have exhibit any significant turning behavior or preference to a higher or lower concentration of said protein gradient, for both concentrations (Fisher’s PLSD Test: 5: p = 0.7805, 2: p = 0.7066). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning for the Sema3a 5 µg/ml condition (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning p = 0.5778, Low Conc. Turning and No Preferential Turning p = 0.7805). For Sema3a 2 µg/ml condition, type I SG neurites preferred to turn to either higher or lower concentration protein gradient rather than have no preferential turning (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning p = 0.0963, Low Conc. Turning and No Preferential Turning p = 0.0440*). Type II SG neurites seemed to prefer a lower concentration gradient of Sema3a at 5 µg/ml than a higher concentration gradient, however the result was not statistically significant (Fisher’s PLSD Test: 5: p = 0.1305). There was no difference between the amount of type II SG neurites that turned towards either a higher or lower protein concentration gradient for the 2 µg/ml condition (Fisher’s PLSD Test: 2: p = 0.8506). There was a significant difference between the amount of type II SG neurites that turned towards a lower protein concentration gradient at the 5
μg/ml Sema3a condition rather than one that had no preferential turning (Fisher’s PLSD Test: 5: p = 0.0302*). At the 2 μg/ml Sema3a condition majority of type II SG neurites had exhibited a turning behavior, either to a higher or lower protein concentration gradient, rather than not having any preferential turning (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning p = 0.0658, Low Conc. Turning and No Preferential Turning p = 0.0963). Refer to figure 9 for the Sema3a 5 μg/ml conditions and figure 10 for the Sema3a 2 μg/ml in the appendix.

6. Type 1 vs. Type 2 SG neurite preferential turning to High Sema5a Protein Gradient Concentration vs. Low Sema5a Protein Gradient Concentration vs. No Sema5a Protein Gradient Concentration Turning Preference.

Type 1 SG neurites cultured on plates with protein depletion gradient of Sema5a did not have exhibit any significant turning behavior or preference to a higher or lower concentration of said protein gradient, for both concentrations (Fisher’s PLSD Test: 5: p = 0.3515, 2: p = 0.2847). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning for both the Sema3a 5 μg/ml and 2 μg/ml condition (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning: 5: p = 0.8146, 2: p =0.5910, Low Conc. Turning and No Preferential Turning: 5: p = 0.4833, 2: p = 0.5910). For type II SG neurites, there was no significant preferential turning difference a higher or lower protein concentration gradient for both the 5 μg/ml and 2 μg/ml condition (Fisher’s PLSD Test: 5: p= 0.1661, 2: p = 0.5910). There was a significant difference between the amount of type II SG neurites that turned towards a lower protein concentration gradient at the 5 μg/ml Sema5a
condition rather than one that had no preferential turning (Fisher’s PLSD Test: 5: p = 0.0142*). At the 2 µg/ml Sema3a condition majority of type II SG neurites had exhibited a turning behavior, either to a higher or lower protein concentration gradient, rather than not having any preferential turning (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning: 2: p = 0.0187*, Low Conc. Turning and No Preferential Turning: 2: p = 0.0643). Refer to figure 11 for the Sema5a 5 µg/ml conditions and figure 12 for the Sema5a 2 µg/ml in the appendix.

7. Type 1 vs. Type 2 SG neurite preferential turning to High LN Protein Gradient Concentration vs. Low LN Protein Gradient Concentration vs. No LN Protein Gradient Concentration Turning Preference.

Type 1 SG neurites cultured on plates with protein depletion gradient of LN showed turning preference to a higher protein concentration gradient of LN rather than a lower concentration of LN, for the 5 µg/ml condition, and a opposite effect at the 2 µg/mL condition, where type I SG neurites preferred a lower LN concentration gradient (Fisher’s PLSD Test: 5: p = 0.0431*, 2: p = 0.0807). There was however no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning for the LN 5 µg/ml condition, while the 2 µg/ml condition had a significant difference between lower LN concentration gradient and no preferential turning, but no significant difference between higher LN concentration gradient and no preferential turning (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning: 5: p = 0.2643, 2: p=0.5837, Low Conc. Turning and No Preferential Turning: 5: p = 0.3373, 2: p = 0.0224*). For type II SG neurites, there was no significant preferential
turning difference a higher or lower protein concentration gradient, for both the 5 µg/ml and 2 µg/ml conditions (Fisher’s PLSD Test: 5: p= 0.4229, 2: p = 0.4123). There was no significant difference, for both the 5 µg/ml and the 2 µg/ml conditions, between the amount of type II SG neurites that exhibited no preferential turning effect and those that preferred either a higher or lower LN concentration gradient (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning: 5: p = 0.1146, 2: p =0.5837, Low Conc. Turning and No Preferential Turning: 5: p = 0.4229, 2: p = 0.1754). Refer to figure 13 for the LN 5 µg/ml conditions and figure 14 for the LN 2 µg/ml in the appendix.

8. Type 1 vs. Type 2 SG neurite preferential turning to High BSA Protein Gradient Concentration vs. Low BSA Protein Gradient Concentration vs. No BSA Protein Gradient Concentration Turning Preference.

Type 1 SG neurites cultured on plates with protein depletion gradient of BSA showed no turning preference to either a higher or lower BSA concentration gradient, for both 5 µg/ml and the 2 µg/ml conditions (Fisher’s PLSD Test: 5: p = 0.5101, 2: p = 0.7949). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning for BSA 5 µg/ml and 2 µg/ml condition (Fisher’s PLSD Test: High Conc. Turning and No Preferential Turning: 5: p = 0.2378, 2: p =0.3013, Low Conc. Turning and No Preferential Turning: 5: p = 0.0688, 2: p = 0.4369). For type II SG neurites, there was no significant preferential turning difference a higher or lower protein concentration gradient, for both the 5 µg/ml and 2 µg/ml conditions (Fisher’s PLSD Test: 5: p= 0.8950, 2: p = 0.3013). There was no significant difference, for both the 5 µg/ml and the 2 µg/ml conditions, between the
amount of type II SG neurites that exhibited no preferential turning effect and those that
preferred either a higher or lower BSA concentration gradient (Fisher’s PLSD Test: High
Conc. Turning and No Preferential Turning: 5: p = 0.7918, 2: p = 0.5837, Low Conc.
Turning and No Preferential Turning: 5: p = 0.8950, 2: p = 0.8965). Refer to figure 15 for
the BSA 5 µg/ml conditions and figure 16 for the BSA 2 µg/ml in the appendix.
IV. DISCUSSION

The results showed that there was a significant difference between type 1 SGNs termination on the PLL OFF stripes vs. Sema3a ON stripes (5 µg/ml and 2 µg/ml conditions), Sema5a ON Stripes (5 µg/ml and 2 µg/ml conditions), and LN ON stripes (5 µg/ml and 2 µg/ml conditions), where type 1 SG neurites preferred terminating on the PLL OFF stripes. Type 2 SGNs followed suit on Sema5a and LN ON stripes (for 5 µg/ml and 2 µg/ml conditions respectively) however; for Sema3a, type 2 SGNs favored Sema3a ON stripes over PLL off stripes. As expected, type I and type II SG neurites did not show significant difference in BSA ON or OFF stripe neurite termination. There was no significance found in type I or type 2 SG neurite turning behavior towards a higher or lower protein gradient concentration for Sema3a, Sema5a, or BSA protein gradients. There was a significant preference of type I SGNs towards a higher LN concentration gradient versus a lower LN concentration gradient at the 5 µg/mL condition. Type II SG neurites for Type II SGNs make up only a small part of the SGN population neonatally due to the massive type II SGN apoptosis that occurs postnatally. Thus, and significant differences between the number of neurites between type I and type II SGNs can be attributed to the fact that there are less type II SGN outgrowth in the first place.

At the moment little is known about the effects that ECM molecules like Laminin or Semaphorin proteins like Sema3a or Sema5a will have on Type 2 SGN OHC outgrowth, turning, and termination. Little is also known about whether a diffused concentration gradient of these proteins will have any or no effect of either type I or type II SGN turning behavior. No studies have been done to look at Sema3a or Sema5a as potential guidance cues for both type I and type II SGNs, and therefore conclusions about
these results will be based on expression patterns of Sema3a and Sema5a in and around
the sensory epithelium, as well as mechanisms by which growth cones mediate turning
and termination responses.

1. Type I and II SGN Response to ON vs. OFF stripes of Sema3a, Sema5a, and
Laminin

In this study we showed that type II SG neurites exhibit a turning patterned
response to Sema3a, Sema5a, and LN protein stripes. This turning is most likely
mediated by changes in the actin-cytoskeleton of SG neurites when induced by the
chemorepulsion or chemoattraction of each respective gradient. SG neurites contain
actin-filled projections called filopodia, which helps to mediate turning in response to a
substrate of physical contact or chemical signaling by extending or retracting the
filopodia. Repellants are thought to cause the loss of actin bundles in the edges of the
growth cone facing the repellant substrate. The side facing away from the substrate will
have an increase of microtubule extensions and turn away from signal. Substrates that are
attractants, induce increase of actin in the growth cone, and the result is turning towards a
signal (Zhou et al., 2005).

It was found that type IIs terminate mostly on Sema3a ON stripes rather than PLL
OFF stripes, the opposite response as seen for type I SG neurite termination. There is
Sema3a expression in the ganglion and in the sensory epithelium just outside the hair cell
area at E15.5 (See appendices, figure 2b) as well as expression of its receptor Nrp-1 at E
14.5 (See appendices, figure 3a). It is the expression outside of the ganglion that is the
most important for guiding neurites. There could be a mechanism by which Sema3a helps
with growth cone collapse of type II SGNs when they reach their outer hair cell targets, especially since its expression location is in a closer vicinity to OHCs versus IHCs. This could be the reasoning as to why type I SG neurites are repulsed by Sema3a ON stripes, to repel type I SGNs away from OHCs and towards their IHCs targets. Sema3a has been known to interact with Plexin A-3, which is heavily expressed in the spiral ganglion (see appendices, figure 3d).

For Sema5a and LN, type IIs terminated mostly on OFF PLL stripes vs. Sema5a and LN stripes respectively. Sema5a is expressed just below the sensory epithelium but not in the ganglion, and its receptor, Plexin B-3, is also expressed in the areas surrounding the sensory epithelium (see appendices, figure 3c). This expression pattern could explain why Sema5a acts as a repellant to both type I and type II SGNs, to provide repulsion to drive them away from the area below the sensory epithelium and towards their respective hair cell targets.

In regards to LN, a study done by Evans et al., in 2007 looked at LN stripes effects on rat neonatal SGN explants outgrowth, specifically acting as a chemorepellent having the majority of SGN explants landing on the ON stripes; however, type I SGN population were not differentiated from type II SGN population and therefore no conclusions could be made about differences in termination patterns between type I and type II SGNs. LN was shown in that study to act as an inhibitory substrate at concentrations around 10 and 5 µg/ml (Evans et al., 2007), which is what was observed in this study. The murine cochlea has been stained for LN and it was observed in the membrane surrounding the developing SG somata and dendritic processes start from P0 and increasing at P2 (Cosgrove and Rodgers, 1997). LN seems to be in the right place at
the right time for influencing outgrowth of developing SG neurites, perhaps playing a role in vivo in the separation of type I and type II SGNs when they are guided towards their cell targets. As far as spatially, LN expression below the IHCs may help repel type II neurons away to their more distant OHC targets. In addition, Aletsee et al., (2001) found radial outgrowth of SG neurites on uniform surfaces coated with LN. However, the growth that was observed not unpatterened, and exhibited turning, typical of a neurite response to a guidance cue or guidance cue like substrate.

2. Type I and II SGN response to High vs. Low Concentration of Sema3a, Sema5a, and Laminin

There was no significant difference in neurite turning preference for Sema3a or Sema5a. However, with LN, there was a preference for type Is towards a higher LN concentration at the 5 µg/mL condition, but an almost opposite effect at the 2 µg/mL. LN, which is expressed beneath the IHCs and therefore can promote and direct SG dendrite growth towards the organ of Corti. There may be a mechanism as to slow down neurite growth so as to allow time for the growth cone filopodia of the type I SGNs to reach and find their targets (Evans et al., 2007). Since, LN is expressed beneath the IHCs more than it is expressed by the OHCs, this could explain why type Is favor a higher concentration, since it might signal them to their IHC targets. With Sema3a at 5 µg/ml, there seemed to be a preference of higher protein concentration gradient for type II SGNs, rather than a lower protein concentration gradient. This was not found to be significant, but perhaps future repeat studies there will be a significance found. As expected, there was no preferential turning of either type I or type II SGNs to a higher or lower BSA
concentration gradient. BSA is not expressed in the rat or mice cochlea, and is a neutral compound, therefore shouldn't have any kind of guidance cue like functionality for either type I or type II SGNs turning behavior.

3. Implication for inner ear and potential guidance cue mechanism

The patterned growth and turning of both type I and type II SGNs to Sema3a, Sema5a, and Laminin, indicate these substrates' involvement of guiding SG neurites to the organ of Corti in vivo. Future studies can include embryonic protein expression assay, looking at particular protein expression patterns in different areas of prenatal sensory epithelium during auditory development. This will give a clearer picture as to the timeline of expression of these potential guidance cues, and at what timepoint during auditory development are these guidance cues upregulated and downregulated to help with axonal guidance and innervation. It would be also interesting to study the distribution of Sema3a, Sema5a, and LN on type I or type II SG neurites during growth into and through the organ of Corti. Sema5a and its interactions with CSPGs and HSPGs can be incorporated in a separate study, to see if the conversion of Sema5a from a chemorepellant to a chemoattractant when incubated with HSPGs is possible, and whether that will change the effects of how type I and type II SGNs terminate and whether they will prefer a higher or lower Sema5a concentration gradient.

More studies need to be done to optimize peripherin staining, using different species of antibody. In addition, other potential guidance cues can be looked at using this protein depletion gradient system, specifically morphogenic factors like BMPs, WNTs, and SHH, to see if there is any significant effects of how these proteins gradient will
affect turning behavior of type II SGNs. Comprehensive and collective studies are key in elucidating the mechanisms behind type II SGNs mysterious yet precise and specific axonal guidance path and OHC innervations.
Figure 1. Effects of 5 µg/mL Sema3a stripes on type I vs. type II SG neurite termination. Explants were grown on alternating stripes of Sema3a vs. PLL. Number of neurites terminating on Sema3a and PLL were counted and compared by Fisher’s PLSD Test. 8 SGN explants were used for each condition. The majority of type 1 SG explants had more neurites terminating on PLL as opposed to Sema3a (p = 0.0039*). Type 2 SG explants had the opposite effect, where more type 2 SG neurites terminated on the Sema3a ON stripes rather than PLL OFF stripes (p < 0.0008*). Data are the mean ± SEM; *p < 0.05.
Figure 2. Effects of 2 µg/mL Sema3a stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of Sema3a vs. PLL. Number of neurites terminating on Sema3a and PLL were counted and compared by Fisher’s PLSD Test. 7 SGN explants were used for each condition. The majority of type 1 SG explants had more neurites terminating on PLL as opposed to Sema3a (p = 0.0005*). Type 2 SG explants had the opposite effect, where more type 2 SG neurites terminated on the Sema3a ON stripes (p ≤ 0.0008*). Similar results when compared to Sema3a 5 µg/mL conditions respectively. Data are the mean ± SEM; *p < 0.05.
Figure 3. Effects of 5 µg/mL Sema5a stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of Sema5a vs. PLL. Number of neurites terminating on Sema5a and PLL were counted and compared by Fisher’s PLSD Test. 6 SGN explants were used for each condition. The majority of type 1 SG explants had more neurites terminating on PLL OFF stripes as opposed to Sema5a ON stripes (p = 0.0393*). Type 2 SG explants had the same effect, where more type 2 SG neurites terminated on the PLL OFF stripes as opposed to the Sema5a ON stripes (p <= 0.0393*). Data are the mean ± SEM; *p < 0.05.
Figure 4. Effects of 2 µg/mL Sema5a stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of Sema5a vs. PLL. Number of neurites terminating on Sema5a and PLL were counted and compared by Fisher’s PLSD Test. 9 SGN explants were used for each condition. The majority of type 1 SG explants had more neurites terminating on PLL as opposed to Sema5a (p < 0.0001*). Type 2 SG explants had the same effect, where more type 2 SG neurites terminated on the PLL OFF stripes than on the Sema3a ON stripes (p = 0.0001*). Similar results when compared to Sema5a 5 µg/mL conditions respectively. Data are the mean ± SEM; *p < 0.05.
**Figure 5.** Effects of 5 µg/mL LN stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of LN vs. PLL. Number of neurites terminating on LN and PLL were counted and compared by Fisher’s PLSD Test. 6 SGN explants were used for each condition. The majority of type 1 SG explants had more neurites terminating on PLL as opposed to LN ($p = 0.0148^*$). Type 2 SG explants had the same effect, where more type 2 SG neurites terminated on the PLL OFF stripes than on the LN ON stripes ($p = 0.1611$). Data are the mean ± SEM; *$p < 0.05$. 

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**Interaction Bar Plot for Neurite Number**

*Effect: Protein Conc. Gradient*

*Error Bars: ± 1 Standard Error(s)*
**Figure 6.** Effects of 2 µg/mL LN stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of LN vs. PLL. Number of neurites terminating on LN and PLL were counted and compared by Fisher’s PLSD Test. 7 SGN explants were used for each condition. Type 1 SG explants had more neurites terminating on PLL OFF stripes as opposed to LN ON stripes (p < 0.0001*). Type 2 SG explants had the same effect, where type 2 SG neurites preferred to terminate on the PLL OFF stripes than on the LN ON stripes (p < 0.0280*). Data are the mean ± SEM; *p < 0.05.
Figure 7. Effects of 5 µg/mL BSA stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of BSA vs. PLL. Number of neurites terminating on BSA and PLL were counted and compared by Fisher’s PLSD Test. 10 SGN explants were used for each condition. Type 1 SG explants did not have any significant difference in whether neurites terminated on PLL OFF stripes or whether they terminated on BSA ON stripes (p = 0.5502). Type 2 SG explants did not have any significant difference in whether neurites terminated on PLL OFF stripes or whether they terminated on BSA ON Stripes (p = 0.4739). Data are the mean ± SEM; *p < 0.05.
Figure 8. Effects of 2 µg/mL BSA stripes on type I vs. type II SG neurites termination. Explants were grown on alternating stripes of BSA vs. PLL. Number of neurites terminating on BSA and PLL were counted and compared by Fisher’s PLSD Test. 8 SGN explants were used for each condition. Type 1 SG explants did not have any significant difference in whether neurites terminated on PLL OFF stripes or whether they terminated on BSA ON stripes (p = 0.7694). Type 2 SG explants did not have any significant difference in whether neurites terminated on PLL OFF stripes or whether they terminated on BSA ON Stripes (p = 0.7694). This Data are the mean ± SEM; *p < 0.05.
**Figure 9.** Effects of 5 µg/mL Sema3a protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of Sema3a on one side of the SGN explant, and a lower concentration of Sema3a on the opposite side. The number of neurites that turned towards versus away versus no preferential turning were counted and compared by Fisher’s PLSD Test. 8 SGN explants were used per condition. Type 1 SGNs did not significant preference between turning towards a higher or lower concentration of Sema3a (p = 0.7805) There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning (High Conc. Turning and No Preferential Turning p = 0.5778, Low Conc. Turning and No Preferential Turning p = 0.7805). Type II SG neurites seemed to prefer a lower concentration gradient of Sema3a at 5 µg/ml than a higher concentration gradient, however the result was not statistically significant (p = 0.1305). There was a significant difference between the amount of type II SG neurites that turned towards a lower protein concentration gradient at the 5 µg/ml Sema3a condition rather than one that had no preferential turning (p = 0.0302*). Data are the mean ± SEM; *p < 0.05.
Figure 10. Effects of 2 µg/mL Sema3a protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of Sema3a on one side of the SGN explant, and a lower concentration of Sema3a on the opposite side. The number of neurites that turned towards versus away versus no preferential turning were counted and compared by Fisher’s PLSD Test. 7 SGN explants were used per condition. Type 1 SGNs did not significant preference between turning towards a higher or lower concentration of Sema3a (p = 0.7066). Type I SG neurites preferred to turn to either higher or lower protein concentration gradient rather than have no preferential turning (High Conc. Turning and No Preferential Turning p = 0.0963, Low Conc. Turning and No Preferential Turning p = 0.0440*). There was no difference between the amount of type II SG neurites that turned towards either a higher or lower Sema3a concentration gradient (p = 0.8506). Majority of type II SG neurites had exhibited a turning behavior, either to a higher or lower protein concentration gradient, rather than not having any preferential turning (High Conc. Turning and No Preferential Turning p = 0.0658, Low Conc. Turning and No Preferential Turning p = 0.0963). Data are the mean ± SEM; *p < 0.05.
**Figure 11.** Effects of 5 µg/mL Sema5a protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of Sema5a on one side of the SGN explant, and a lower concentration of Sema5a on the opposite side. The number of neurites that turned towards versus away versus no preferential turning were counted and compared by Fisher’s PLSD Test. 6 SGN explants were used per condition. Type 1 SG neurites did not exhibit any significant turning behavior or preference to a higher or lower Sema5a concentration gradient (p = 0.3515). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning (High Conc. Turning and No Preferential Turning: p = 0.8146, Low Conc. Turning and No Preferential Turning: p = 0.4833). For type II SG neurites, there was no significant preferential turning difference a higher or lower Sema5a concentration gradient (p = 0.1661). There were significantly more type II SG neurites that turned towards a lower Sema5a concentration gradient than those that did not have any preferential turning (p = 0.0142*). Data are the mean ± SEM; *p < 0.05.
Figure 12. Effects of 2 µg/mL Sema5a protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of Sema5a on one side of the SGN explant, and a lower concentration of Sema5a on the opposite side. The number of neurites that turned towards versus away versus no preferential turning were counted and compared by Fisher’s PLSD Test. 9 SGN explants were used per condition. Type 1 SG neurites did not exhibit any significant turning behavior or preference to a higher or lower Sema5a concentration (p = 0.2847). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning (High Conc. Turning and No Preferential Turning: p = 0.5910, Low Conc. Turning and No Preferential Turning: p = 0.5910). For type II SG neurites, there was no significant preferential turning difference a higher or lower Sema5a concentration gradient (p = 0.5910). Majority of type II SG neurites exhibited a turning behavior, either to a higher or lower protein concentration gradient, rather than not having any preferential turning (High Conc. Turning and No Preferential Turning: p = 0.0187*, Low Conc. Turning and No Preferential Turning: p = 0.0643). Data are the mean ± SEM; *p < 0.05.
Figure 13. Effects of 5 µg/mL LN protein depletion gradient on type I and type II SGN neurites cultured on plates, with a higher concentration of LN on one side of the SGN explant, and a lower concentration of LN on the opposite side. The number of neurites that turned towards versus away versus no preferential turning, were counted and compared by Fisher’s PLSD Test. 6 SGN explants were used per condition. Type I SGN neurites cultured on plates with protein depletion gradient of LN showed turning preference to a higher protein concentration gradient of LN rather than a lower concentration of LN (p = 0.0431*). There was however no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning for the LN 5 µg/ml condition (High Conc. Turning and No Preferential Turning: p = 0.2643, Low Conc. Turning and No Preferential Turning: p = 0.3373). For type II SGN neurites, there was no significant preferential turning difference a higher or lower LN concentration gradient (p= 0.4229). There was no significant difference between the amount of type II SGN neurites that exhibited no preferential turning effect and those that preferred either a higher or lower LN concentration gradient (High Conc. Turning and No Preferential Turning: p = 0.1146, Low Conc. Turning and No Preferential Turning: p = 0.4229). Data are the mean ± SEM; *p < 0.05.
Figure 14. Effects of 2 µg/mL LN protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of LN on one side of the SGN explant, and a lower concentration of LN on the opposite side. The number of neurites that turned towards versus away versus no preferential turning, were counted and compared by Fisher’s PLSD Test. 7 SGN explants were used per condition. Type I SG neurites cultured on plates with protein depletion gradient of LN showed turning preference to a lower protein concentration gradient of LN rather than a higher concentration of LN (p = 0.0807). Type I SG neurites had a significant preference towards a lower LN concentration gradient rather than no preferential turning, but they did not have a significant preference for a higher LN concentration gradient versus no preferential turning (High Conc. Turning and No Preferential Turning: p = 0.5837, Low Conc. Turning and No Preferential Turning: p = 0.0224*). For type II SG neurites, there was no significant difference between preferential turning towards a higher or lower LN concentration gradient (p = 0.4123). There was no significant difference between the amount of type II SG neurites that exhibited no preferential turning effect and those that preferred either a higher or lower LN concentration gradient (High Conc. Turning and No Preferential Turning: p = 0.5837, Low Conc. Turning and No Preferential Turning: p = 0.1754). Data are the mean ± SEM; *p < 0.05.
Figure 15. Effects of 5 µg/mL BSA protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of Sema3a on one side of the SGN explant, and a lower concentration of Sema3a on the opposite side. The number of neurites that turned towards versus away versus no preferential turning, were counted and compared by Fisher’s PLSD Test. 10 SGN explants were used per condition. Type 1 SG neurites cultured on plates with protein depletion gradient of BSA showed no turning preference to either a higher or lower BSA concentration gradient (p = 0.5101). There was also no significant difference between a higher or lower protein concentration gradient preference and that of no preferential turning (High Conc. Turning and No Preferential Turning: p = 0.2378, Low Conc. Turning and No Preferential Turning: p = 0.0688). For type II SG neurites, there was no significant preferential turning difference a higher or lower BSA concentration gradient (p= 0.8950). There was no significant difference between the amount of type II SG neurites that exhibited no preferential turning effect and those that preferred either a higher or lower BSA concentration gradient (High Conc. Turning and No Preferential Turning: p = 0.7918, Low Conc. Turning and No Preferential Turning: p = 0.8950). Data are the mean ± SEM; *p < 0.05.
Figure 16. Effects of 2 μg/mL BSA protein depletion gradient on type I and type II SG neurites cultured on plates, with a higher concentration of BSA on one side of the SGN explant, and a lower concentration of BSA on the opposite side. The number of neurites that turned towards versus away versus no preferential turning, were counted and compared by Fisher’s PLSD Test. 8 SGN explants were used per condition. Type 1 SG neurites cultured on plates with protein depletion gradient of BSA showed no turning preference to either a higher or lower BSA concentration gradient (p = 0.7949). There was also no significant difference between a higher or lower BSA concentration gradient preference and that of no preferential turning (High Conc. Turning and No Preferential Turning: p =0.3013, Low Conc. Turning and No Preferential Turning: p = 0.4369). For type II SG neurites, there was no significant preferential turning difference a higher or lower protein concentration gradient (p = 0.3013). There was no significant difference between the amount of type II SG neurites that exhibited no preferential turning effect and those that preferred either a higher or lower BSA concentration gradient (High Conc. Turning and No Preferential Turning: p =0.5837, Low Conc. Turning and No Preferential Turning: p = 0.8965). Data are the mean ± SEM; *p < 0.05.
APPENDICES

High Conc.

Figure 1a: Depletion Gradient Striping Pattern from Folch et al., 2008.

Low Conc.

Figure 1b: Microfluidic Channels apparatus from Folch et al., 2008

Reservoir

Channels
Figure 2a: Sema3A expression E15.5, C57BL/6 Mouse Cochlea, Image: Head_914_1_4C (genepaint.org) Genepaint Set ID: HB547

Figure 2b: Sema5a expression E14.5, C57BL/6, Mouse Cochlea, Image: Embryo_C1046_8_2A (genepaint.org) Genepaint Set ID: ST55

Legend:
SE = Sensory Epithelium
SG = Spiral Ganglion
Figure 3a: Nrp-2 Expression
E14.5, C57BL/6, Mouse Cochlea,
Image: Embryo_B813_4_2C
(genepaint.org)
Genepaint Set ID: EB1966

Figure 3b: Nrp-1 Expression
E14.5, C57BL/6, Mouse Cochlea,
Image: Embryo_S308_5_2A
(genepaint.org)
Genepaint Set ID: ES1008

Figure 3C: Plexin B-3 Expression
E14.5, C57BL/6, Mouse Cochlea,
Image: Embryo_C1619_1_5C
(genepaint.org)
Genepaint Set ID: ST82

Figure 3D: Plexin A-3 Expression
E14.5, C57BL/6, Mouse Cochlea,
Image: Embryo_EX132_2_2C
(genepaint.org)
Genepaint Set ID: ST57
REFERENCES


Tashiro, K., Sephel, G., Weeks, B., Sasaki, M., Martin, G., Kleinman, H. and Yamada,


