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Responses of Type II Spiral Ganglion Neurites to Stripes of Laminin and Fibronectin

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Michael L. Sung

Committee in charge:

> Professor Allen F. Ryan, Chair
> Professor William B. Kristan
> Professor Nicholas C. Spitzer

2011

The thesis of Michael L. Sung is approved and it is acceptable in quality and form for publication on microfilm or electronically:

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<div align="right">Chair</div>

University of California, San Diego

2011

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LIST OF SYMBOLS

ECM   extracellular matrix
FN   fibronectin
IHC   inner hair cell
LN   laminin
SG   spiral ganglion
OHC   outer hair cell
P(age)   postnatal day
PLL   poly-L-lysine

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ACKNOWLEDGEMENTS

ABSTRACT OF THE THESIS

Responses of Type II Spiral Ganglion Neurites to Stripes of Laminin and Fibronectin

by

Michael L. Sung

Master of Science in Biology

University of California, San Diego, 2011

Professor Allen F. Ryan, Chair

The goal of this study was to evaluate the role of extracellular matrix proteins in the guidance of type II spiral ganglion neurons. To accomplish this, we investigated the effect of stripes of extracellular matrix proteins laminin (LN) and fibronectin (FN) on the outgrowth of mouse type II SG neurites *in vitro*. Little is known about the guidance cues that direct the type II SG neurites to the outer hair cells in the Organ of Corti. However, laminin and fibronectin have both been found to be expressed in tracts leading to and

beneath the Organ of Corti, and are thus potential candidate guidance molecules. Growth

cone targeting was determined for fibers extending from spiral ganglion explants cultured

on stripes of LN and FN in alternation with poly-L-lysine (PLL) or on alternating stripes

of LN and FN. Type II SG neurites grown on alternating stripes of LN and PLL tended

to avoid LN at all but the lowest concentration. Neurites tended to avoid high

concentrations of FN relative to PLL, and showed no preference at intermediate and

lower FN concentrations. When explants were grown on alternating stripes of LN and FN

there was no significant difference in the number of type II neurite endings on either

ECM molecule. The results indicate that both LN and FN can serve as guidance cues for

type II spiral ganglion neuron neurites. Moreover, the responses of type II neurites differ

from those of type I neurites, and may contribute to differences in the innervation

patterns of the two neurite types.

I. INTRODUCTION

1. Spiral ganglion neurons and hair cells

In the mammalian auditory system, sensory hair cells in the Organ of Corti transmit auditory information from the cochlea to the central nervous system via spiral ganglion neurons. Spiral ganglion neurons are divided into two types: type I spiral ganglion, which constitute approximately 90-95% of the neuron population, are bipolar and myelinated; Type II neurons, which constitute approximately 5-10% of the neuron population, are pseudomonopolar and unmyelinated (Berglund and Ryugo, 1991; Spoendlin, 1973; Perkins and Morest, 1975). The adult mouse cochlea contains approxiamtely 800 inner hair cells (IHC) that are each exclusively innervated by up to twenty type I SG neurons. There are approximately 2600 outer hair cells (OHC) which are innervated only by the type II SG neurons, with each neuron contacting 10-20 OHCs (Keithley and Feldman, 1982; Burda and Branis, 1988; Pujol et al. 1998). The OHCs are unique sensory cells in that they are mechanically active. Their membranes incorporate a molecule, prestin, which alters its shape in response to changes in membrane current. The combined action of many molecules results in changes in the length of the cell. This activity amplifies mechanical input to the OHCs by 100-1000 fold, and this energy is fed back into the basilar membrane. OHC amplification is the basis for the high sensitivity and frequency tuning in the mammalian cochlea. (for review see Ashmore, 2008)

Type I and type II SG neurons are thought to have distinct functions, with type I SG neurons being the primary cells that transmit auditory information from the

cochlea to the central nervous system, and type II SG neurons are thought to participate in a feedback loop that regulates the OHC amplifier.

2. SG neuron projections

The type I SGN dendrites reach the IHCs via a straight path from the SG through the osseous spiral lamina. Each SGN dendrite typically synapses with a single IHC. As noted above, up to 20 dendrites contact each IHC. Type II SGNs project through the osseous spiral lamina, pass under the IHCs, and project across the organ of Corti to the region of the OHCs. They then turn toward the cochlear base and project for approximately 500-600 um, gradually ascending within the organ until they reach the bases of the OHCs. Each dendrite then branches and contacts many OHCs (for review see Rubel and Fritzsch, 2002).

3. SG neuron innervation patterns during development

The development of SG afferent organization has been studied in a number of mammalian systems. These studies suggest that overgrowth followed by pruning of afferent neurites is the primary mechanism leading to the mature SG neuron - hair cell organization.

In the gerbil, neurites were reported to contact both IHCs and OHCs at birth, but by P6 type I and type II neurites had segregated to synapse with inner and outer hair cells respectively (Echteler, 1992). During afferent reorganization in the first postnatal week, there is also a 27% decrease in the number of gerbil spiral ganglion cells (Echteler and Nofsinger, 2000). Unpublished data suggest that most of this die-off represents type II

SGNs (Barclay, Ryan and Housley). In the rat, similar changes were observed between P0 and P16 to the pattern of hair cell innervation, with the majority of reorganization occurring between P9 and P12 (Lenoir, 1980). Huang et al. (2007) described development of innervation in the mouse Organ of Corti. Between E18 and P0 both type I and II afferent fibers contacted both inner and outer hair cells in the developing Organ of Corti. At P3 outer spiral bundles were seen to innervate the outer hair cells. From P3 to P6 the SG afferent neurites went through a process of neuronal remodeling. By P6 the inner hair cells were innervated by type I fibers with limited number of processes contacting the first row of outer hair cells while type II fibers formed synapses with all three rows of outer hair cells. All of these changes occur prior to the onset of cochlear function, which is initiated a few days after cochlear innervation reaches an approximately adult configuration.

Altricial mammals such as mice, rats and gerbils are good model systems for studying the hair cell innervation because most cochlear development occurs postnatally. (Rubel and Fritzsch, 2002). In contrast, the development of cochlear innervation occurs prior to the onset of function at about 25 weeks of gestation in humans. (Depres, 1994; Pujol, 1995)

4. Peripherin as marker for type II neurons

Past studies have used various methods including electron microscopy and horseradish peroxidase staining to track SG neurite outgrowth toward hair cells during cochlear development (Perkins and Morest, 1975; Echteler, 1992; Simmons, 1994). However, these methods were unable to distinguish between type I and type II neurons.

More recently, peripherin (a type III intermediate filament) was found to be highly enriched in type II SG neurites and to serve as a marker to distinguish this subpopulation of SG neurons.

Immunistochemical studies have used anti-peripherin to stain specifically for type II SG neurites. Hafidi et al. (1993) observed peripherin immunoreactivity starting from E20 in spiral ganglion cell bodies, which was reduced to a small population of neurons corresponding to type II SG neurons in adult rats. In the human fetal cochlea, peripherin staining was limited to a small population of ganglion neurons and their processes that extended to the outer hair cells (Despres et al., 1994). *In vitro* studies have also confirmed the use of peripherin as a marker for type II SG neurites (Mou et al., 1998; Reid et al., 2004) in the mouse. In the rat, type I SGNs upregulate peripherin *in vitro*, making this marker inappropriate for this species in culture. (Ryan and Housley unpublished observations)

5. Type I versus II spiral ganglion neurons

In addition to differences in synaptic targets and physiology, the distribution of type I and type II connections differ along the length of the cochlea. Inner hair cells receive three times as many synapses from type I neurons in the basal turn and connections between type II neurons and outer hair cells are twice as numerous in the apical turn (Rubel and Fritzch, 2002).

There is variation even within the small population of type II neurons from different turns of the cochlear. Fechner et al. (2001) reported that the majority of basal type

II fibers synapse with the first row of OHCs and apical type II afferents synapse with all three rows of OHCs.

6. Extracellular matrix molecules

Extracellular matrix molecules are thought to be involved in neuron guidance. Among them are two well characterized molecules: laminin and fibronectin.

Laminin is cross-shaped molecule composed of alpha, beta and gamma chain subunits. LN molecules bind to form polymer networks that are a major component of the basement membrane. The different domains of laminin are associated with various aspects of laminin function including neurite guidance and cell adhesion (Luckenbill-Edds, 1997). The peptide sequence IKVAV in the E8 domain has been reported to be involved in neurite outgrowth and cell attachment associated with LN (Tashiro, 1989). The LN isoform LN-1 (the isoform used in this study) was isolated from the Engelbreth-Holm-Swarm mouse sarcoma cells (Timpl and Rohde, 1979).

Fibronectin is a 500 kDa dimeric glycoprotein made up of two subunits linked by disulfide bonds. Each subunit is composed of three types of multiple elements called "repeats." In humans, alternative splicing of FN mRNA is responsible for producing as many as twenty types of FN. This allows for FN isoforms with different cell adhesive, ligand binding and solubility qualities (Pankov and Yamada, 2002). The RGD sequence, within the type III fibronectin repeat, is an integrin recognition sequence and is thought to be involved in cell adhesion (Ffrench-Constant, 1995).

7. Laminin and fibronectin expression in the inner ear

Immunohistochemical studies have found LN and FN to be expressed in the avian and mammalian cochleas and located in close proximity to SG afferent processes coinciding temporally with the innervation of the Organ of Corti.

In the mouse cochlea, LN staining is observed in the basement membrane associated with the developing spiral ganglion cell bodies and SG dendritic fibers starting from P0 and increasing at P2 before decreasing at P14 (Cosgrove and Rodgers, 1997). Laminin staining is also present beneath the inner hair cells (Cosgrove and Rodgers, 1997). Positive laminin staining was found in the adult guinea pig in areas surrounding the spiral ganglion cells and neurites (Takahashi and Hokunan, 1992). Laminin immunoreactivity has been observed around the chick basal papilla (avian structure synonymous with the mammalian Organ of Corti) directly in the path of cochlear ganglion fibers (Hedmond and Morest, 1991).

Fibronectin is widely expressed by P2 but was markedly reduced in the area surrounding the SG cell bodies by P4 (Cosgrove and Rodgers, 1997). Fibronectin is expressed in the spaces beneath the inner and outer hair cells, apparently in linear tracks beneath the sensory cells (Woolf et al., 1992). Also, fibronectin is colocalized with laminin in the chick cochlea during innervation of the basal papilla (Hedmond and Morest, 1991).

8. Laminin and fibronectin in neurite guidance

LN is a permissive growth substrate for neurites in cell culture models (Manthorpe et al., 1983; Rogers et al., 1983; Hall et al., 1987). Also, LN coated beads

serve as an attractant to extending neurites *in vitro* in addition to increasing the rate of growth cone extension (Kuhn et al., 1995). Stripes of LN direct the outgrowth of a variety of different types of neurons *in vitro* (Hammarback et al., 1985; Turney and Bridgmann, 2005; Clark et al., 1993).

FN is not as conducive to neurite outgrowth as LN (Manthorpe et al., 1983; Hall et al., 1987). Chick retinal and spinal cord neurons do not to extend processes on FN but are able to grow on LN (Rogers et al., 1983). Chick DRG neurites also reduce their growth rate when encountering a FN coated bead on a homogenous LN surface (Kuhn et al., 1995).

The use of stripe and border assays with different types of neurons has been used to study both affinity for LN or FN and behavior of growth cones at the border between two substrates. DRG growth cones change rate of extension and direction when encountering a LN-FN border (Gomez and Letourneau, 1994). Goldfish retinal axons grow in lanes of LN when presented with alternating stripes of FN and LN (Vielmetter et al., 1990).

LN and FN guide SG neurites *in vitro*. SG neurites were shown to track and preferentially terminate on stripes of LN at relatively high concentrations and avoid LN at relatively low concentrations (Evans et al., 2007). When SG neurites were grown on alternating stripes of FN and PLL, the neurites preferentially terminated on stripes of PLL at relatively high concentrations of FN. In a competition assay between LN and FN, SG neurites preferentially terminated on LN stripes.

9. SG neurite responses to laminin and fibronectin

Both laminin and fibronectin influence SG neurites *in vitro*. Laminin induces a dose-dependent increase in the length and number of spiral ganglion neurites *in vitro* (Aletsee et al., 2001). LN induces an increase in SG neurite length mediated by a pathway involving the G protein Ras and the mitogen-activated protein kinase (MAPK) Erk. However, LN-induced increase in SG neurite number was found only to signal through (MAPK) Erk (Aletsee et al., 2002). The Rho GTPases (Rho, Rac and Cdc42) are also thought to be involved in LN mediated SG neurite outgrowth (Brors et al., 2003). SG neurties branch, turn or stop when approaching a FN border (Aletsee et al., 2000).

10. Stripe Assays

Stripe assays were developed to assess the ability of potential guidance molecules to influence extending axons *in vitro*. Bonhoeffer and colleagues created the technique to examine guidance cues involved in the avian retinal system. The assay was used to assess the outgrowth of chick retinal ganglion cells given the choice between opposing stripes of anterior and posterior of tectal membranes (Walter et al., 1987). Previous versions of the stripe assay used crude membrane fractions (Walter et al., 1987; Vielmetter and Suermer, 1989) or cells transfected with a guidance molecule (Nguyen-Ba-Charvet et al., 2001). Purified proteins have also been used to create stripes using methods similar to the protocol used in this study. A silicon mask was used to create alternating stripes of protein on the bottom of a plastic culture well, which were subsequently blocked with bovine serum albumin (Vielmetter et al., 1990; Nguyen-Ba-Charvet et al., 2001; Jain et al., 2004).

Stripe assays allow for the study of the guidance cues present during the development of both the central and peripheral nervous systems (Mann et al., 1998; Savaskan et al., 1999; Knoll *et al*., 2001; Knoll *et al*., 2006) and more specifically in the auditory system (Evans et al., 2007).

Evans and colleagues (2007) showed that both laminin and fibronectin can influence the outgrowth of total spiral ganglion neuron population *in vitro*. The majority of neurons studied were presumably Type I neurons, since they outnumber type II neurons by more than 10 to 1 at the age studied. However, the authors noted a heterogeneous response of neurites grown on intermediate concentrations of LN versus PLL. Different neurites from the same explants were observed to track either on stripes of LN or PLL. It was postulated that this inhomogeneous response was due to the two populations of SG neurons. However, this study addressed the total SG neurites population but did not investigate the affect of LN and FN on type II SG neurites in particular. The affect of LN and FN specifically on the guidance of type II SG neurons remains unknown.

The objective of this study was to evaluate whether the ECM glycoproteins laminin and fibronectin influence the outgrowth of type II spiral ganglion neurites and to compare their relative attractive or replusive effects. The ultimate goal is to better understand the molecular cues that mediate the unique pattern of type II SG neurite extension toward the OHCs in the developing Organ of Corti.

II. MATERIALS AND METHODS

1. Preparing the tissue culture plates

The stripe molds were generated by applying a silicone rubber (Sylgard 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 vacuum before being cut into 4 x 4 mm pieces, sterilized with 100% ethanol and air dried inside a sterile hood overnight. The silicone molds was placed groove side down in the center of a 24 well cell culture plate (Costar, Corning Inc.). The first ECM protein solution was applied to the top of the mold and the ECM protein solution was drawn into the channels by suction applied along the bottom edge of the mold. After filling the channels with protein, the bottom edge of the mold was sealed with ECM protein solution to prevent evaporation of fluid from the channels during overnight incubation at 37°C with the silicone mold in place.

After incubation, the mold channels were washed twice with phosphate buffered saline (PBS). If making alternating stripes of ECM molecules, stripes were blocked with 2% bovine serum albumin at 37°C for 2h. Subsequently, the mold was removed from the culture plate and the well was washed with PBS. Each well was filled with 250 μl of 5 μg/ml PLL solution (Sigma-Aldrich) or a second ECM protein solution and incubated at 37°C for 1 hour. Dulbecco's modified eagle medium (DMEM), (Gibco) was used to washed the wells twice before being filled with 170 μl of primary attachment medium: DMEM, 10% fetal bovine serum (Sigma-Aldrich), 25mM HEPES buffer (Gibco) and 300 U/ml penicillin (Sigma-Aldrich).

FN from human plasma (Sigma-Aldrich) and LN1 from Engelberth-Holms sarcoma (Sigma-Aldrich) were used in these experiments.

To eliminate coating order as a variable influencing the guidance of the type II neurites, the experiments comparing LN versus FN were performed twice with alternating the coating of the ECM glycoproteins.

2. Spiral ganglion dissection

Neonatal (P2) 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. SG tissue was cut into three equal pieces separating the apical, middle and basal turns of the spiral ganglion before being placed in primary attachment medium.

3. Tissue culture

After dissection, explants were incubated at $37\,^{o}C$ in primary attachment medium for 24h. Then the culture medium was replaced with serum-free maintenance media (DMEM (Gibco), 25mM HEPES buffer (Gibco), 6mg/ml glucose (Gibco), 300 units/ml penicillin (Sigma-Aldrich) and 30 μl/ml N2-supplement (Gibco). Maintenance medium was supplemented with 50 ng/ml of recombinant BDNF (R&D Systems) to support type II SG neurite outgrowth. Explants were cultured in a humidified incubator with 5% CO_2 and $37\,^{o}C$ for 72h.

4. Immunohistochemistry

SG explants were fixed for 20 min at room temperature with 4% paraformaldehyde, then washed twice with phosphate buffered saline (PBS) (Gibco). The fixed explants were permeabilized for 10 minutes with 5% Triton X-100 (Sigma-Aldrich) and washed twice with PBS before blocking with 5% donkey serum to reduce non-specific staining (Sigma-Aldrich). Type II SG neurites were labeled using a polyclonal chicken antibody against the 57 kDa intermediate filament peripherin (1:1000; Thermo Scientific). LN and FN stripes were labeled with polyclonal rabbit antibodies against LN, FN (1:400; Sigma-Aldrich). Explants were incubated overnight at $4^{o}C$ in primary antibody and were later washed twice with PBS. Samples were incubated for 2.5 hours with fluorescein isothiocyanate (FITC) or Texas-red (TR) conjugated secondary antibodies (1:100; Jackson Immunoresearch) against either chicken or rabbit primary antibodies. Incubation without primary antibody was used as a negative control to verify staining specificity.

5. Data Analysis

For each turn of the cochlea, at least 8 SG explants were evaluated and at least 28 explants were evaluated for each concentration of ECM molecule. Digital images were obtained on an fluorescence microscope (Olympus FSX100). Images were stitched together and optimized using Adobe Photoshop (Adobe Systems Inc.). The neurite terminations were marked and counted to determine substrate preference. Statview 5.0 was used to perform non-parametric Mann-Whitney U test and Kruskal-Wallis multivariate test to determine statistical significance.

III. RESULTS

1. Type II SG neurites on stripes of LN versus PLL

Type II SG neurites cultured on plates with alternating stripes of LN versus PLL tended to have significantly more neurites ending on PLL than on LN, when the results from all cochlear turns were combined. At thefour highest LN concentrations (80, 40, 20 and 10 μg/ml), more neurites ended on PLL (5 μg/ml) than on LN (Mann-Whitney U test: 80: $p < 0.0300$*; 40: $p < 0.0443$*; 20: $p < 0.0009$**; 10: $p < 0.0204$*; 5: $p < 0.3068$). Neurites from many explants tracked on PLL stripes when alternated with 80, 40 and 20 μg/ml LN stripes (Figure 6).

Type II SG neurites from the three cochlear terms (apical, medial and basal) were also analyzed to determine if turn origin of the explant influenced the distribution of neurite termination. The majority of explants from all turns at all tested concentrations had more neurites terminating on PLL as opposed to LN. However, these results did not reach the level of statistical significance in most cases. The results from the apical turn are as follows: (Mann-Whitney U test: 80 μg/ml: $p < 0.1178$; 40 μg/ml: $p < 0.1409$; 20 μg/ml: $p < 0.2505$; 10 μg/ml: $p < 0.0703$; 5 μg/ml: $p < 0.4429$). The results from the medial turn are as follows: (Mann-Whitney U test: 80 μg/ml: $p < 0.0455$*; 40 μg/ml: $p < 0.3246$; 20 μg/ml: $p < 0.0269$*; 10 μg/ml: $p < 0.2702$; 5 μg/ml: $p < 0.9164$). The results from the basal turn are as follows: (Mann-Whitney U test: 80 μg/ml: $p < 0.3425$; 40 μg/ml: $p < 0.1580$; 20 μg/ml: $p < 0.0327$*; 10 μg/ml: $p < 0.1842$; 5 μg/ml: $p < 0.2855$; Figures 1-5). There was no evidence that any individual turn showed a different tendency than any other turn.

2. Type II SG neurites on stripes of FN versus PLL

When the data from all cochlear turns were combined, type II SG neurites cultured on stripes of FN versus PLL preferentially terminated on PLL at 80 and 40 µg/ml FN, while at lower FN concentrations neurites did not display a preference for either FN or PLL (Mann-Whitney U test: 80: $p < 0.0405$*; 40: $p < 0.0409$*; 20: $p < 0.3741$; 10: $p < 0.0639$; 5: $p < 0.0631$; Fig. 12).

As with LN, the behavior of type II SG neurites from the three cochlear terms (apical, medial and basal) were separately analyzed to determine if turn origin of the explant influenced the distribution of neurite termination on alternating stripes of FN and PLL. The majority of explants from all turns at all concentrations had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. The results from the apical turn are as follows: (Mann-Whitney U test: 80 µg/ml: $p < 0.3044$; 40 µg/ml: $p < 0.3075$; 20 µg/ml: $p < 0.4683$; 10 µg/ml: $p < 0.2899$; 5 µg/ml: $p < 0.1580$). The results from the medial turn are as follows: (Mann-Whitney U test: 80 µg/ml: $p < 0.2568$; 40 µg/ml: $p < 0.2568$; 20 µg/ml: $p < 0.4683$; 10 µg/ml: $p < 0.2708$; 5 µg/ml: $p < 0.2253$). The results from the basal turn are as follows: (Mann-Whitney U test: 80 µg/ml: $p < 0.1451$; 40 µg/ml: $p < 0.1451$; 20 µg/ml: $p < 0.8843$; 10 µg/ml: $p < 0.2899$; 5 µg/ml: $p < 0.1891$; Fig. 7 -11). While not significant, the trend of the data suggests no differences between cochlear turns in the response to FN.

3. Competition assay: LN versus FN

Type II neurites were grown on alternating stripes of LN and FN in order to test which ECM molecule was more conducive to neurite outgrowth. The coating

concentration 80 µg/ml was selected because type II neurites were more often observed on PLL than on LN or FN at this concentration in LN versus PLL and FN versus PLL experiments. Type II SG neurites were cultured on alternating stripes of LN and FN showed no preference for either substrate. There was no significant difference in the number of neurite endings on either LN or FN regardless of whether LN was coated first (Mann-Whitney U test: $p < 0.166$; Fig. 13) or FN was the first stripe laid down (Mann-Whitney U test: $p < 0.566$; Fig. 14).

IV. DISCUSSION

In a prior study (Evans et al., 2007) the effects of stripes of extracellular matrix proteins LN and FN on the outgrowth of neonatal rat SGN neurites were analyzed. However, that study analyzed the behavior of the total population of SGN, and did not differentiate between type I and type II neurites. Moreover, at the age evaluated, type I neurites outnumber type II neurites by approximately 10 to 1. Consequently, no conclusion could be drawn about the behavior of the small subpopulation of type II neurons.

At present, little is known about the effect of extracellular matrix proteins on the outgrowth of type II neurons during the innervation of the cochlea. As mentioned above, the morphology, innervation pattern and the biological function of type I and type II within the cochlea differ significantly. Due to these biological differences between the two types of neurons within the cochlea, the aim of this study was to analyze the outgrowth pattern of type II SGN in an alternative choice assay of the extracellular matrix protein LN and FN *in vitro* and compare the results to the prior study (Evans et al., 2007). The same experiments were conducted in order to determine if there was difference in the behavior of type I and type II SG neurons. Since there are very few type II neurons compared to type I neurons in the cochlea, the results of the previous study most likely represents the behavior of type I neurons in this alternative stripe assay, although the possibility of differential survival of neurons or differential projection of neurites from the two types of neurons must be considered.

1. Type II SGN respond to stripes of ECM molecules versus PLL

In the present study, we demonstrated that type II SG neurites cultured on plates with alternating stripes of LN versus PLL had significantly more neurites ending on PLL than on LN. At all but the lowest LN concentrations (80, 40, 20 and 10 µg/ml), more neurites ended on PLL than on LN.

Furthermore, we demonstrated a similar type II neuron response to FN versus PLL *in vitro* in an alternative choice assay. Type II SG neurites cultured on stripes of FN versus PLL preferentially terminated on PLL at 80 and 40 µg/ml FN, while at lower FN concentrations neurites did not display a preference for either substrate. Moreover, we analyzed the growth behavior of type II neurons from the different cochlear turns (apical, medial and basal). In all cases we observed more type II neurons terminating on PLL than on FN or LN stripes, respectively. We did not observe a difference in type II neuron behavior within the different cochlear turns and within all turns we also observe more type II neurons terminating on PLL than on FN or LN, respectively. However, in most cases the difference did not reach statistical significance. This may reflect an insufficient number of samples when the data were divided by turn.

It could be argued that the preference of PLL versus FN and LN is due to a slower growth rate of type II neurons on the PLL surface compared to the ECM molecules. Therefore, the type II SGN would spend more time on the PLL surface and be found more often on the PLL substrate. However, neurite tracking along the stripes as observed in many cases cannot be explained by different growth rates and implies that the direction of the projection of the type II neurons was influenced by the stripes.

Another explanation for a differential distribution of the type II SGN endings on stripes is branching. If the substrate on one stripe increased branching of the neurites in response to the substrate, more type II neurites might be found on that particular stripe. In fact, there is an increase in the number of spiral ganglion neurites from explants on uniform surfaces with increasing amounts of LN (Aletsee et al., 2001), indicating more branching and/or enhanced neuronal or neurite survival, although this study was done on the whole SGN population and type II neurites were not analyzed. If increased branching was an explanation for our results, it had to be mediated by PLL. However, we saw no evidence of increased branching on PLL. Moreover, the tracking along PLL stripes as observed for many explants cannot be explained by branching of SGN neurites.

Another possible mechanism for differential growth is enhanced neuronal survival on one substrate. If PLL caused enhanced SGN survival, or if LN or FN enhanced SGN death, this could result in the observation of more fibers on PLL stripes. We have found no evidence in the literature to suggest that PLL enhances neuronal survival. Moreover, uniform surfaces of FN or LN have been shown to enhance the number of SGN neurites considered as a whole population. For these reasons we consider effects on neuronal survival to be unlikely.

Radial outgrowth of SG neurites on uniform surfaces such as LN has been reported (Aletsee et al., 2001). This observation is in contrast with our findings, in which the type II SGN show a different response when the surface is was discontinuous (Fig. 15). This is in line with the findings by Evans et al. (2007) who found the same outgrowth pattern on a surface with a discontinuous stripe pattern. The outgrowth was not radial and unpatterned but showed turning in response to the stripes. This finding implies

that the observed effects on type II SGN neurite tracking and termination are mediated by turning mechanisms. In fact, SG neurites have actin-filled projections called filopodia, which are thought to mediate turning as they do in other neurons. Contact with a substrate border can result in signaling events and result in cytoskeletal changes and induce attraction or repulsion to the substrate. For example, growth cones traveling on LN towards a border with the ECM molecule tenascin, result in repulsive turning of the SGN after filopodial contact of the tenascin border (Gomez et al., 2000). In addition, actin bundles are positioned to play an important role in extension towards or away from a substrate. It is thought that growth cone encounters with a repellant substrate result in focal loss of actin bundles in the edge of the growth cone in the region facing the repellant. This results in the extension of mircotubules on the side where actin bundles remain and induces turning away from the signal. In contrast, focal increase in actin will result in turning toward a signal (Zhou et al., 2005).

2. No preference for LN or FN

In the first part of the study we investigated the effects of the ECM proteins LN and FN on SGN neurite extension against PLL. As mentioned above PLL had significantly more neurites ending on PLL than on LN at all but the lowest LN concentrations (80, 40, 20 and 10 μg/ml), more neurites ended on PLL than on FN at the two highest concentrations (80 and 40 μg/ml). Lower coating concentrations of the ECM proteins (FN: 20, 10 and 5 μg/ml; LN 5 μg/ml) did not display a preference for either LN or FN, respectively.

The observation that there is no preference for either LN or GN is interesting, since the two ECM studied occur in non-identical patterns in the perinatal Organ of Corti (Wolf et al 1992, Cosgrove and Rodgers 1997, Rodgers et al 2001) and is in contrast with the behaviors of the total SGN population (presumably dominated by type I SGN neurites) and neurons in other systems. Evans et al. (2007) demonstrated a preference of presumed type I SGN terminating on LN in the same alternative choice assay we used. Retinal axons preferentially extended on LN (20 μg/ml) versus FN (50 μg/ml) and they found LN to be the most permissive substrate for neurite outgrowth compared to several other tested substrates (Vielmetter et al., 1990). Also chick doral root ganglion neurites cultured on FN preferentially extended in the direction of LN-coated beads (Kuhn et al., 1995).

Since both LN and FN are found beneath the IHCs, type I dendrites may need to discriminate between LN and FN. For type I neurites, LN is attractive at high concentrations while FN is an inhibitory substrate. Since LN is not present under the OHCs, type II dendrites may not need to discriminate between LN and FN.

3. Implications for the inner ear

LN was shown to be a permissive and attractive growth substrate at the highest concentration (80 μg/ml) and inhibitory at lower concentrations (10 and 5 μg/ml) for the total population of SG neurites *in vitro* (Evans et al., 2007). These observations suggested a role for laminin in guiding developing SG neurons to the Organ of Corti *in vivo*.

In contrast, in this study we show that laminin acts as an inhibitory substrate specifically for type II SG neurites at all tested concentrations for which a response was

noted *in vitro*. The fact that type II neurites avoided LN even at a low concentration (10 μg/ml) suggests that type II afferents may be highly sensitive to LN *in vivo*. It has been demonstrated that type I and II neurites occupy separate growth tracts with the type I fibers lying above the type II neurites during the first postnatal week (Huang et al., 2007). In the mouse cochlea (the model used in this study), laminin staining was observed in the membrane surrounding developing spiral ganglion cell bodies and SG dendritic processes starting from P0 and increasing at P2 (Cosgrove and Rodgers, 1997). LN in murine cochlea has the correct temporal and spatial distribution to potentially influence outgrowth of developing SG neurites. LN could play a role *in vivo* for maintaining separation of these two subpopulations of SG neurites until they reach their respective hair cell targets. It would be interesting to study the distribution of LN on type I or type II SG dendritic processes during growth into and through the Organ of Corti. Additionally, avoidance of LN below the IHCs may promote growth of type II neurons toward the more distant OHC region.

For the total population of SG neurites (Evans et al., 2007), FN was shown to be an inhibitory substrate at relatively high concentrations (80 and 40 μg/ml). For type II SG neurites we found that FN acts as an inhibitory substrate at the same concentrations. Fibronectin was expressed in the area surrounding the SG cell bodies by P4 (Cosgrove and Rodgers, 1997) and observed in the spaces beneath the inner and outer hair cells (Woolf et al., 1992). The inhibitory effect of FN could act to slow the growth rate of type II afferents to allow for synapsing with the OHCs. Alternatively, FN beneath the HCs could tend to force type II neurites toward the cells. Also, during afferent innervation of

the OHCs, type II fibers entering the OHC region turn basally before synapsing. This

turning behavior could possibly involve FN expression beneath the OHCs. However,

since FN is distributed along the length of the cochlea, it would have to be expressed in a

gradient or combined with another signal to induce turning. It should be noted that other

molecules have been identified in the developing mouse cochlea. These molecules

include: tenascin, NCAM, L1 and E-cadherin (Whilton et al., 1999). The stripe assay

used in this study could be applied to investigate the attractive or inhibitory effects of

these molecules on guidance of type I and type II SG neurites could help put the role of

LN and FN in context.

APPENDIX
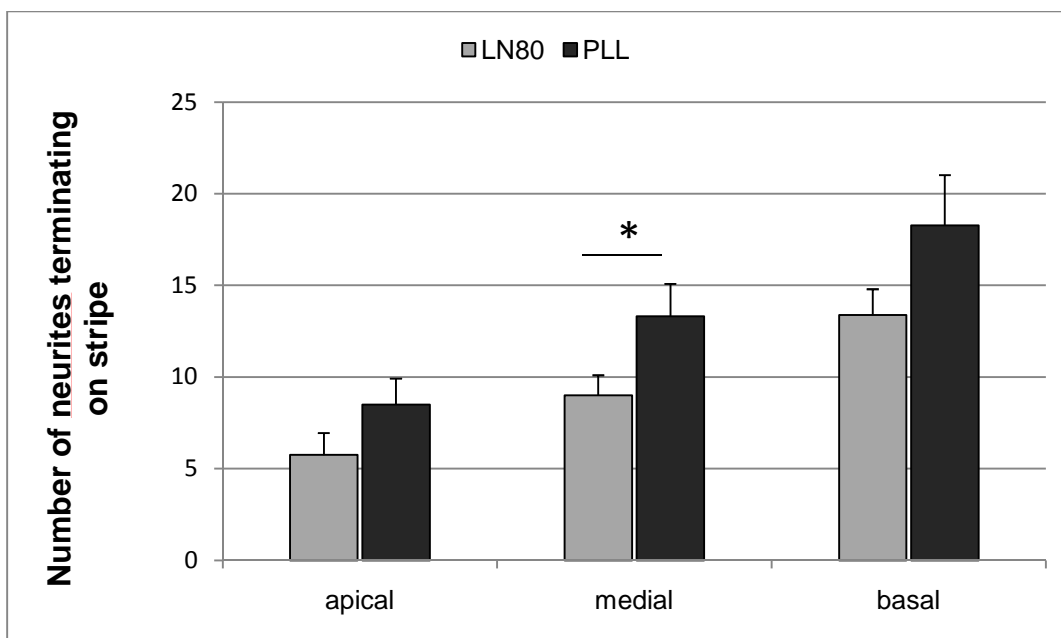
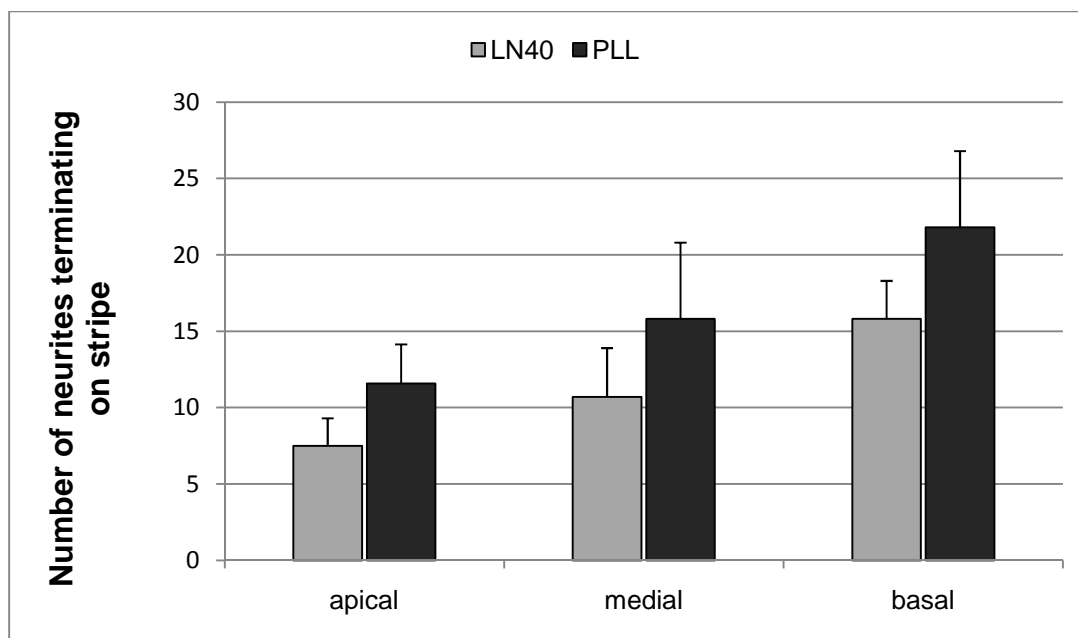**Figure 1.** Effects of 80 µg/mL LN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of LN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 16 and 18 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to LN. However, these results only reached the level of statistical significance for the medial turn (p < 0.0455*). Data are the mean ± SEM; *p < 0.05.

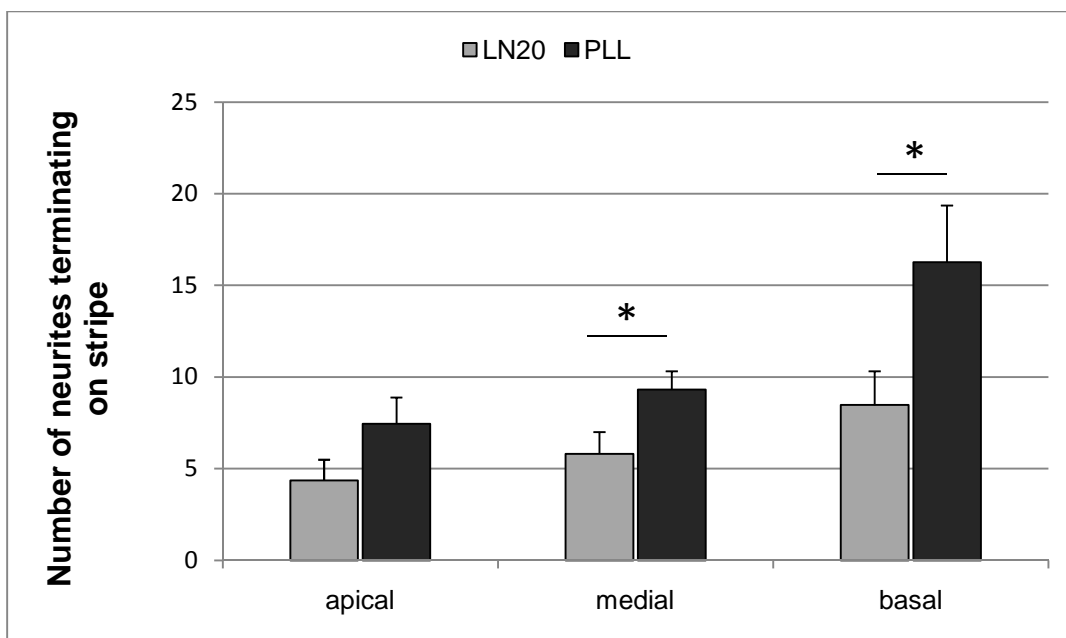**Figure 2.** Effects of 40 µg/mL LN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of LN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 11 and 12 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to LN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
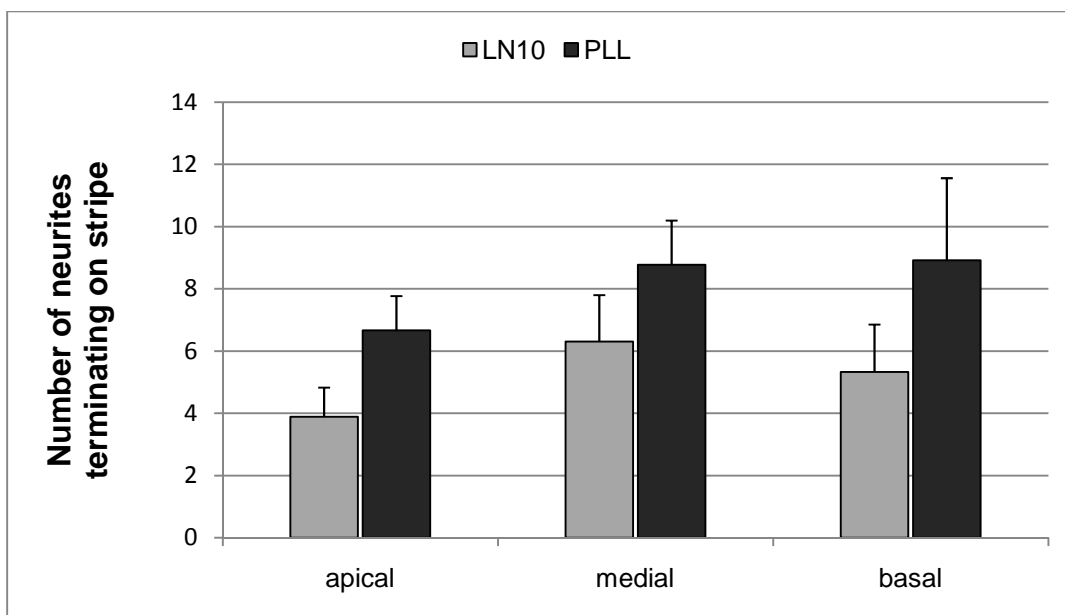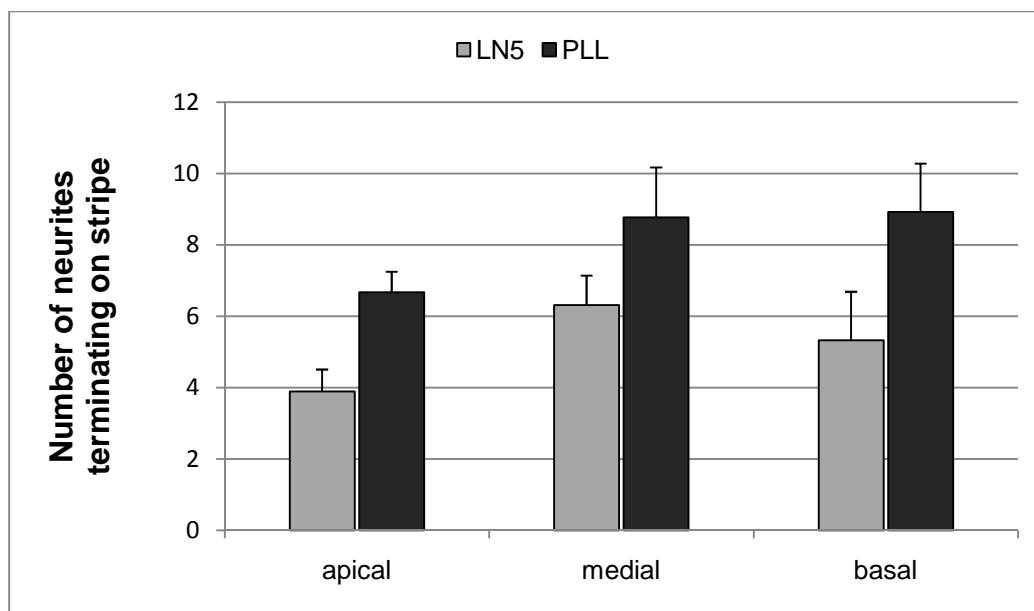
Figure 3. Effects of 20 µg/mL LN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of LN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 11 and 16 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to LN. The results only reach the level of statistical significance for the medial (p < 0.0269*) and basal (p < 0.0327*) cochlear turns. Data are the mean ± SEM; *p < 0.05.

Figure 4. Effects of 10 µg/mL LN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of LN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 10 and 12 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to LN. However, these results were not statistically significant. Data are the mean $\pm$ SEM; *p < 0.05.

Figure 5. Effects of 5 µg/mL LN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of LN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 8 and 15 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to LN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
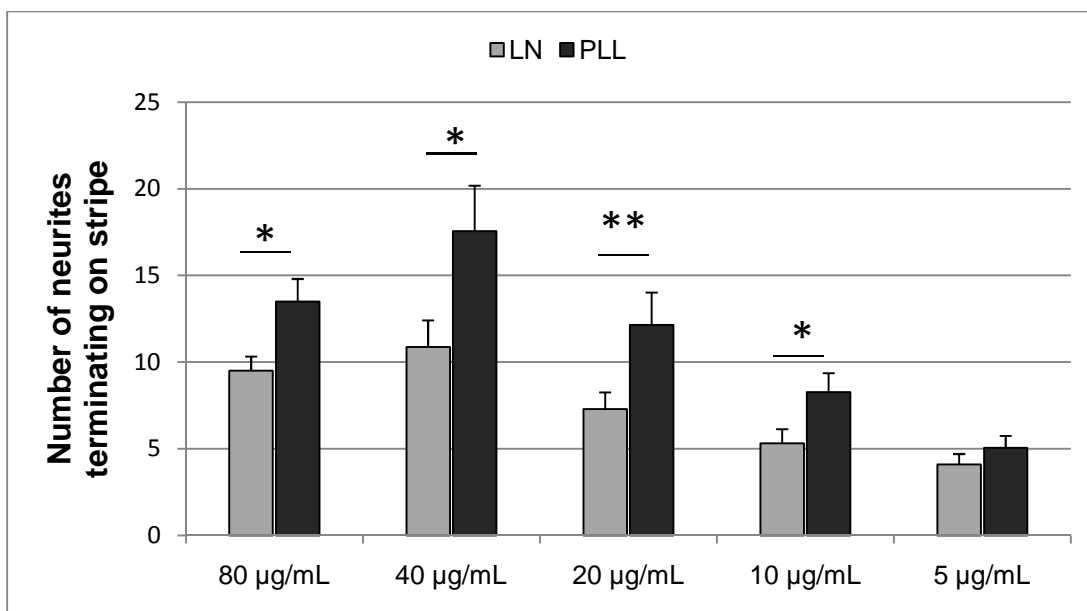
Figure 6. Effects of varying concentration of LN stripes on type II SG neurites from the entire cochela. Explants were grown on alternating stripes of LN (80, 40, 20, 10 or 5 µg/mL) vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 34 and 53 explants were used for each LN concentration. Type II SG neurites preferentially terminated on PLL at 80, 40, 20, 10 µg/mL. (80 µg/mL; p < 0.0300*; 40 µg/mL; p < 0.0443*; 20 µg/mL: p < 0.0009**; 10 µg/mL: p < 0.0204*) At 5 µg/mL, there was no significant difference in the distribution of type II neurite termination. Data are the mean ± SEM; *p < 0.05; **p <0.01.

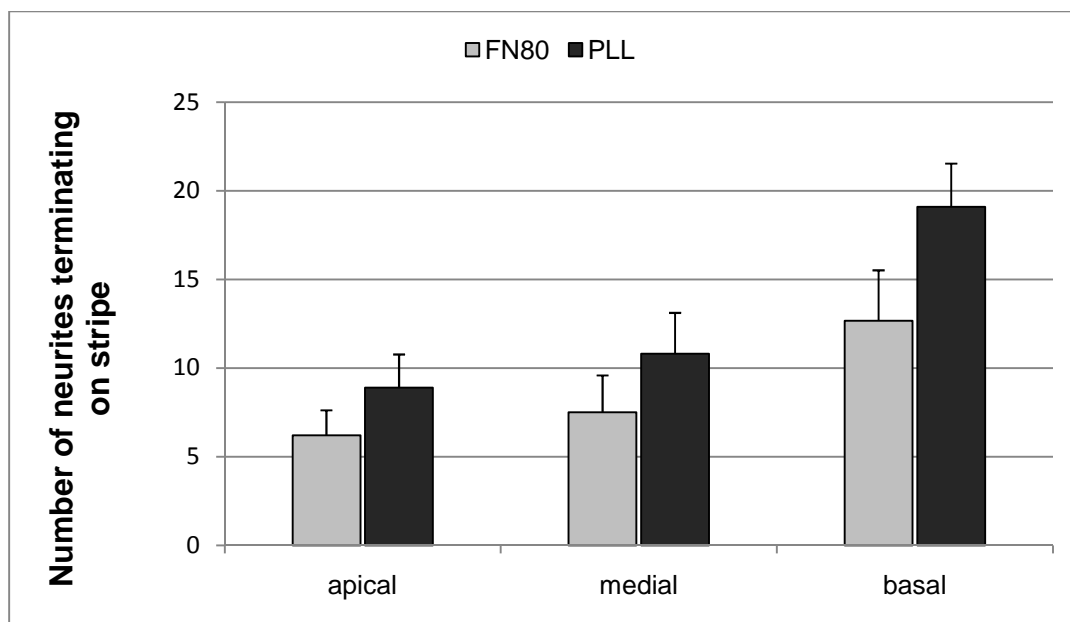**Figure 7.** Effects of 80 µg/mL FN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of FN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 9 and 10 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
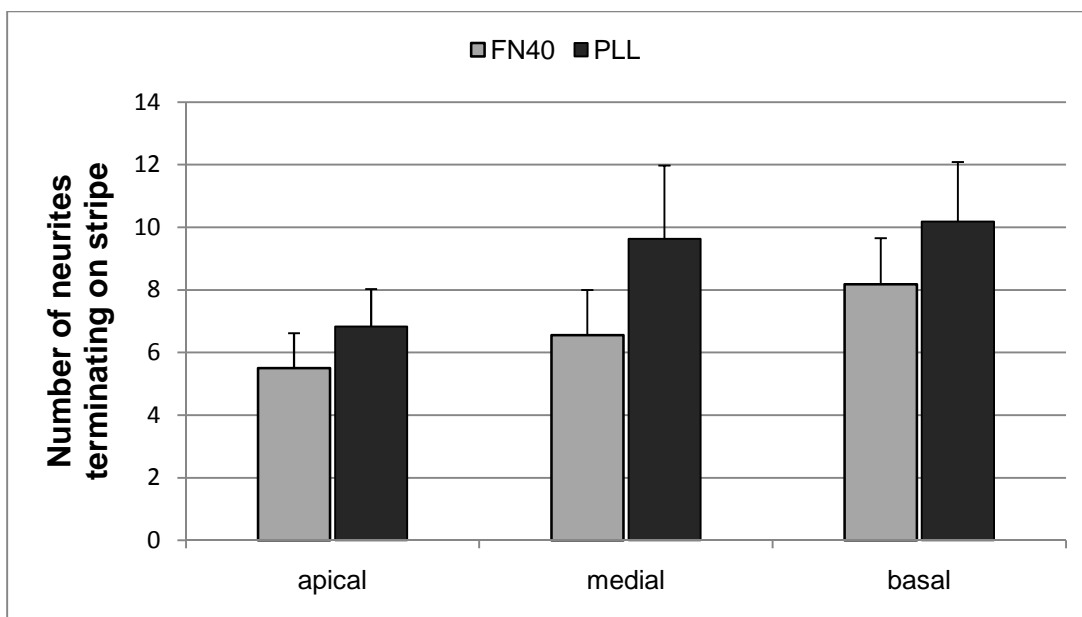
Figure 8. Effects of 40 µg/mL FN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of FN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 11 and 12 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
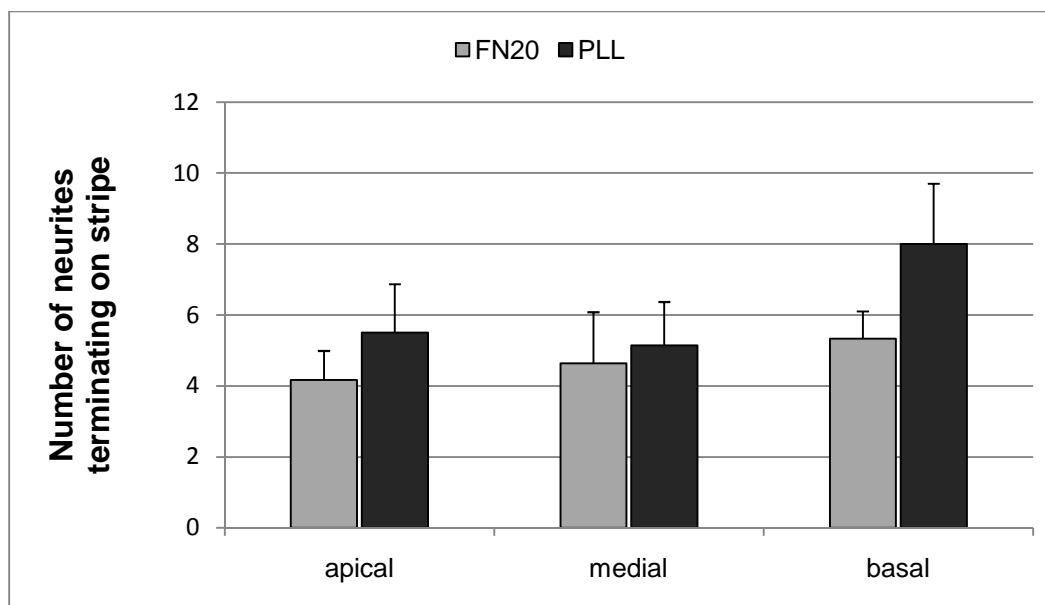
Figure 9. Effects of 20 µg/mL FN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of FN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 12 and 18 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
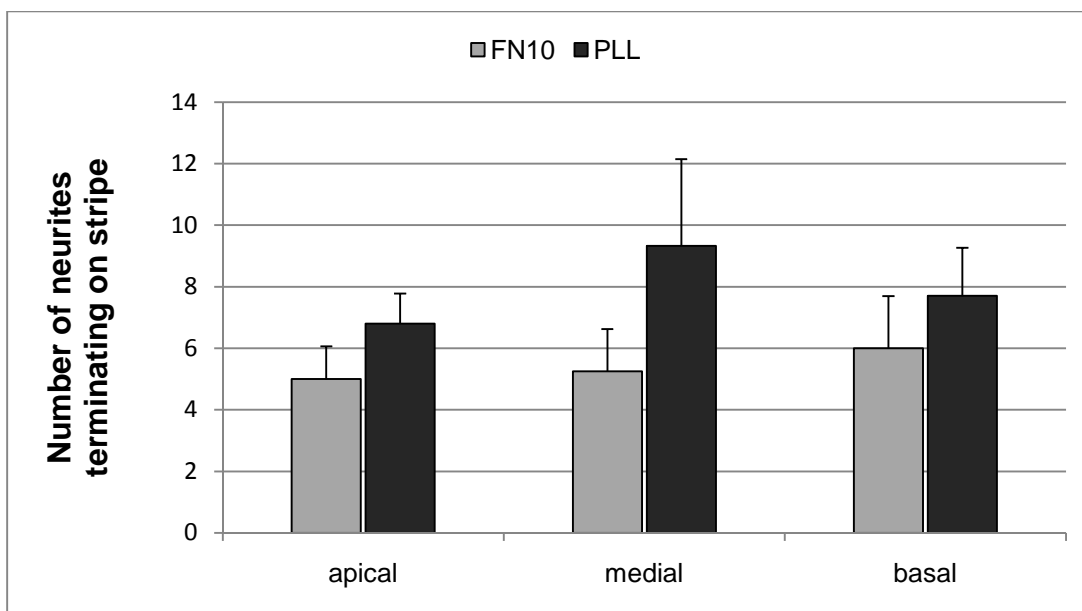
Figure 10. Effects of 10 µg/mL FN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of FN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 10 and 12 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.

Figure 11. Effects of 5 µg/mL FN stripes on type II SG neurites from the three cochlear turns. Explants were grown on alternating stripes of FN vs PLL. Number of neurites terminating on LN and PLL were counted and compared by Mann-Whitney U test. Between 11 and 12 explants were used for each turn of the cochlea. The majority of explants from all turns had more neurites terminating on PLL as opposed to FN. However, these results were not statistically significant. Data are the mean ± SEM; *p < 0.05.
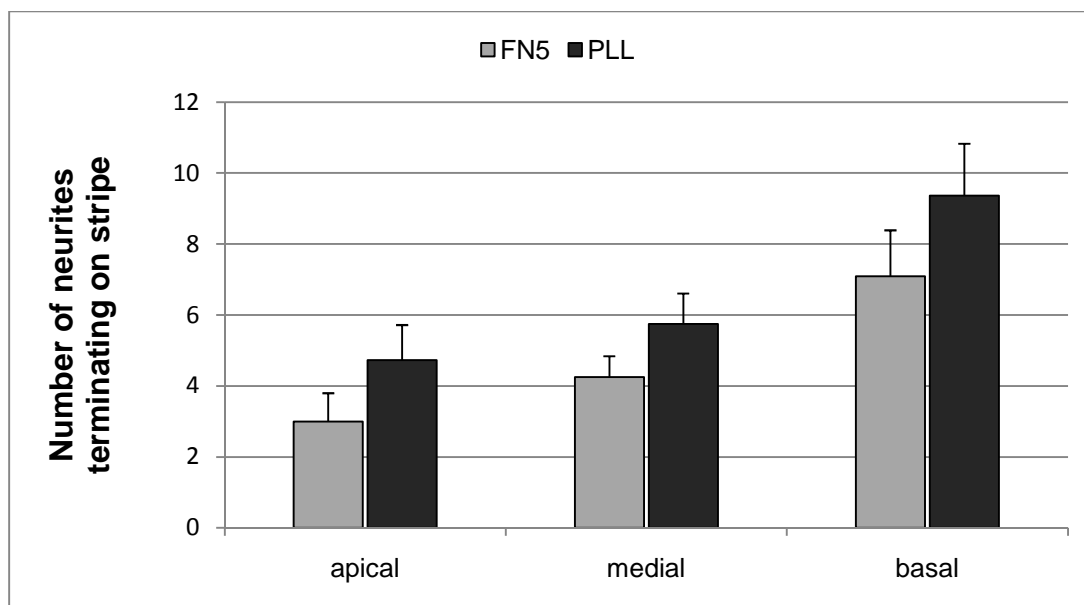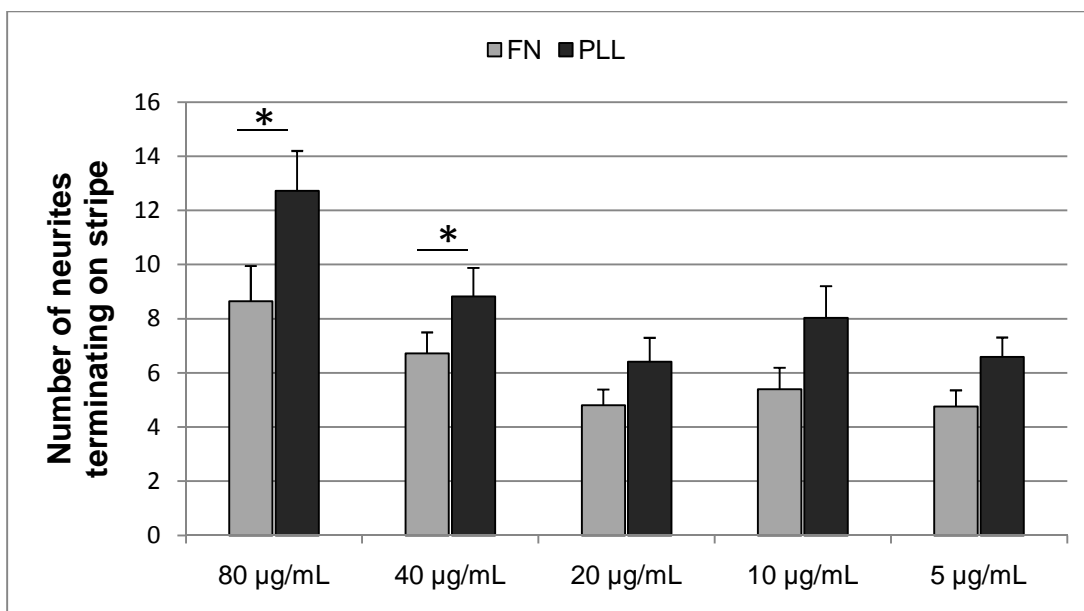
Figure 12. Effects of varying concentration of FN stripes on type II SG neurite termination from the entire cochela. Explants were grown on alternating stripes of FN (80, 40, 20, 10 or 5 µg/mL) vs PLL. Number of neurites terminating on FN and PLL were counted and compared by Mann-Whitney U test. Between 29 and 44 explants were used for each LN concentration. Type II SG neurites preferentially terminated on PLL at 80, 40, 20, 10 µg/mL. (80 µg/mL: $p < 0.0405$*; 40 µg/mL: $p < 0.0409$*) At 20, 10 and 5 µg/mL, there was no significant difference in the distribution of type II neurite termination. Data are the mean ± SEM; *$p < 0.05$.
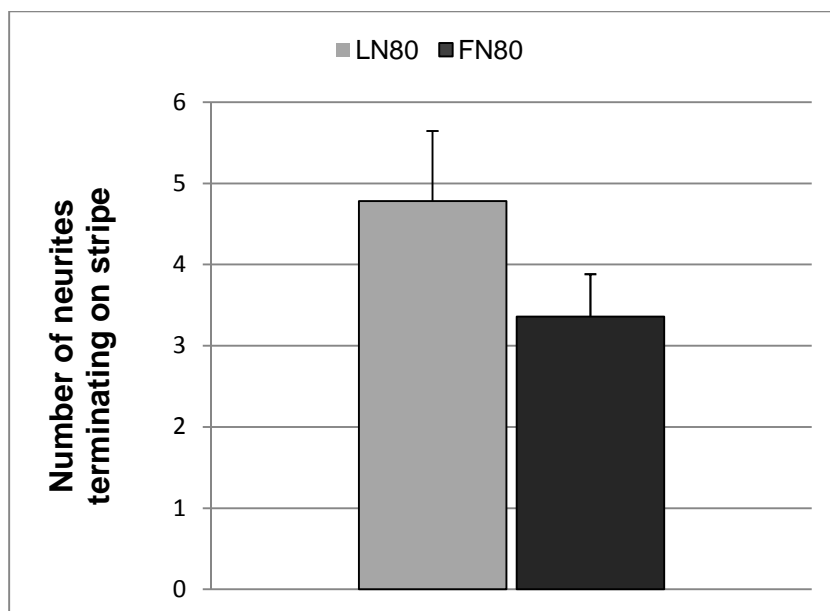
Figure 13. Comparison of the effect of LN 80 µg/mL and FN 80 µg/mL on type II neurite termination. Explants were grown on alternating stripes of LN vs FN. LN was applied first in a stripe pattern and a FN solution was subsequently applied over the stripes. The number of neurites terminating on LN and FN were counted and compared by Mann-Whitney U test. 41 explants were analyzed. The number type II neurites ending on LN or FN was not statistically significant ($p < 0.166$). Data are the mean ± SEM; *$p < 0.05$.

Figure 14. Comparison of the effect of FN 80 µg/mL and LN 80 µg/mL on type II neurite termination. Explants were grown on alternating stripes of FN vs LN. FN was applied first in a stripe pattern and a LN solution was subsequently applied over the stripes. The number of neurites terminating on LN and FN were counted and compared by Mann-Whitney U test. 38 explants were analyzed. The number type II neurites ending on LN or FN was not statistically significant (p < 0.186). Data are the mean ± SEM; *p < 0.05.
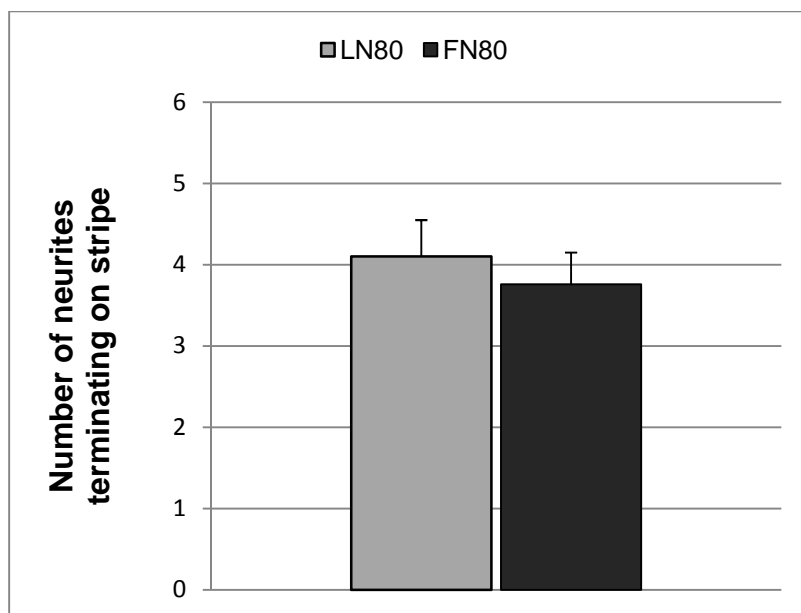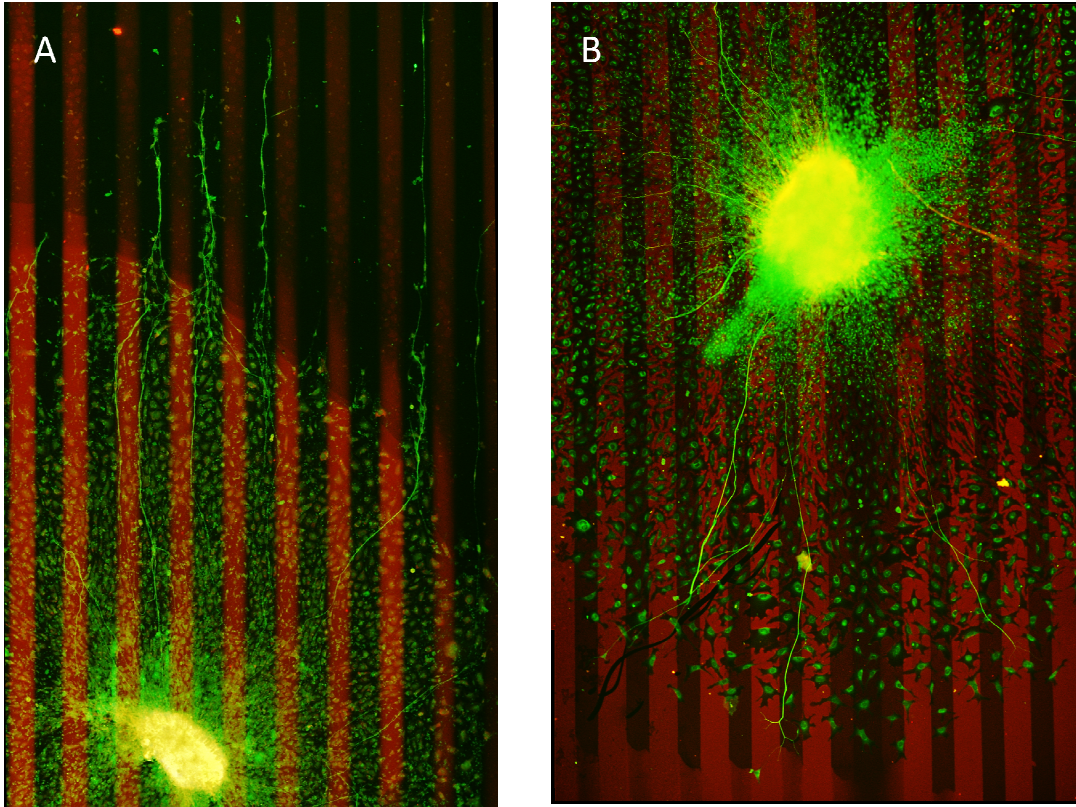
Figure 15. (A) Sample explant displaying neurite preference for PLL when cultured on stripes of LN (20 µg/mL) and PLL. (B) Sample explant displaying no preference for PLL or FN when cultured on alternating stripes of FN (20 µg/mL) and PLL.
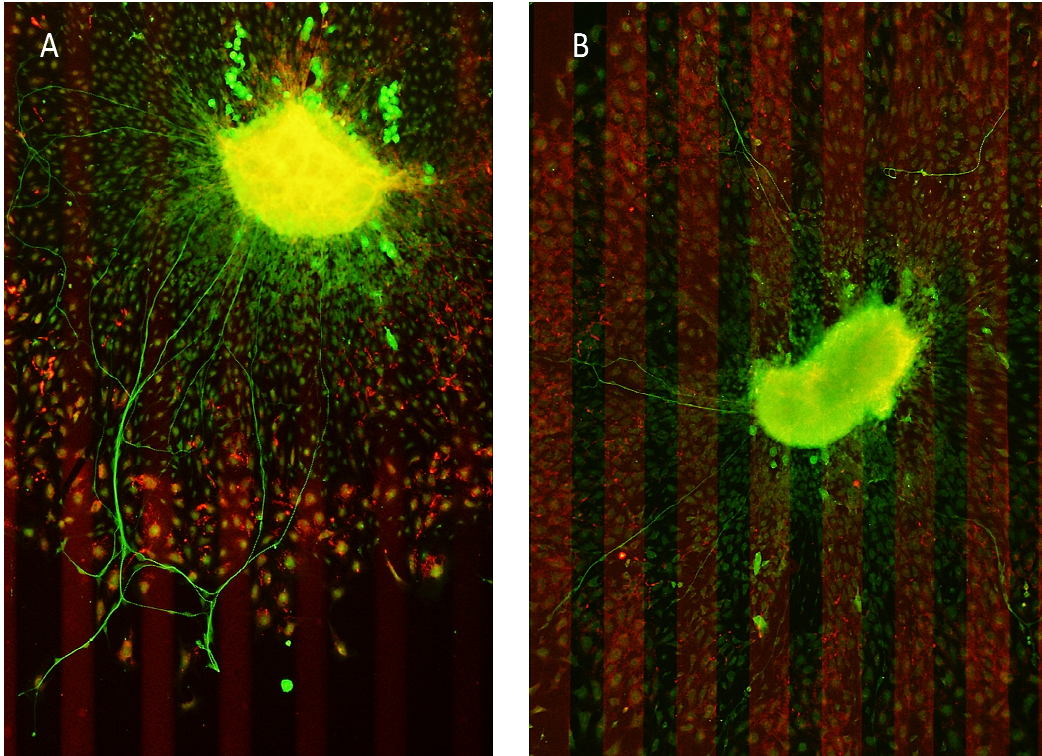
Figure 16. Sample explant cultured on alternating stripes of (A) LN versus FN (LN first stripe) and (B) FN and LN (FN first stripe). Explants grown on alternating stripes of LN and FN did not show preference for either ECM molecule.

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