Laminin and Fibronectin Modulate Inner Ear Spiral Ganglion Neurite Outgrowth in an *In Vitro* Alternate Choice Assay

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Received 22 March 2007; revised 5 May 2007; accepted 11 May 2007

ABSTRACT: **Extracellular matrix (ECM) molecules have been shown to function as cues for neurite guidance in various populations of neurons. Here we show that laminin (LN) and fibronectin (FN) presented in stripe micro-patterns can provide guidance cues to neonatal (P5) inner ear spiral ganglion (SG) neurites. The response to both ECM molecules was dose-dependent. In a LN versus poly-L-lysine (PLL) assay, neurites were more often observed on PLL at low coating concentrations (5 and 10 μg/mL), while they were more often on LN at a high concentration (80 μg/mL). In a FN versus PLL assay, neurites were more often on PLL than on FN stripes at high coating concentrations (40 and 80 μg/mL). In a direct competition between LN and FN, neurites were observed on LN significantly more often than on FN at both 10 and 40 μg/mL. The data suggest a preference by SG neurites for LN at high concentrations, as well as avoidance of both LN at low and FN at high concentrations. The results also support a potential model for neurite guidance in the developing inner ear *in vivo*. LN, in the SG and osseus spiral lamina may promote SG dendrite growth toward the organ of Corti. Within the organ of Corti, lower concentrations of LN may slow neurite growth, with FN beneath each row of hair cells providing a stop or avoidance signal. This could allow growth cone filopodia increased time to sample their cellular targets, or direct the fibers upward toward the hair cells.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 00: 000–000, 2007

Keywords: **extracellular matrix; neurite guidance; spiral ganglion; pattern assay**

INTRODUCTION

Axon and dendrite outgrowth in neurons are influenced by many factors, including the extracellular environment. Extracellular matrix (ECM) molecules and their interaction with cellular receptors, primarily integrins, have been shown to function as neuronal

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Contract grant sponsor: NIH/NIDCD; contract grant number: DC00139.

Contract grant sponsor: Medical Research and Rehabilitation Research and Development Service of the VA.

Contract grant sponsor: German research foundation; contract grant number: EU 120-1/1.

Published online in Wiley InterScience(www.interscience. wiley.com).
DOI 10.1002/dneu.20540

survival signals and cues for axon and dendrite guidance in various populations of neurons (Gomez and Letourneau, 1994; McKerracher et al., 1996).

In the auditory portion of the mammalian inner ear the cochlea, the first neurons of the auditory pathway, reside in the spiral ganglion (SG). The SG is centrally located in the modiolus, while the mechanosensory target cells, inner and outer hair cells (HCs), are located peripherally on the basilar membrane. The ECM molecule laminin (LN), a heterotrimer of α, β, and γ chains, has been identified in the mammalian cochlea in the basement membranes surrounding the developing SG and underlying the basilar membrane (Cosgrove and Rodgers, 1997; Rodgers et al., 2001). During postnatal maturation of the organ of Corti, a change in expression of LN heterotrimer subunits occurs at the same time as the SG dendrites target the HCs (Cosgrove and Rodgers, 1997; Rodgers et al., 2001), suggesting a possible functional linkage of LN to postnatal SG differentiation and dendrite targeting. Another major ECM molecule, the dimeric glycoprotein fibronectin (FN), has also been identified in the mammalian cochlea (Woolf et al., 1992; Cosgrove and Rodgers, 1997). As with LN, a temporo-spatial expression pattern of FN in the path of the SG dendrites was observed in mouse, rat and gerbil (Woolf et al., 1992; Cosgrove and Rodgers, 1997), suggesting a possible function in dendrite targeting. In addition, Toyama et al. (2005) identified various isoforms of the integrin ECM receptor family, previously found to be important in the developing brain, in the perinatal mammalian cochlea. These heterodimeric transmembrane proteins, consisting of one α and one β chain, bind promiscuously to various ECM molecules, promoting adherence and functionally linking them to the actin cytoskeleton and other intracellular effectors.

Homogenous surfaces of LN and FN have been shown to stimulate rat neonatal SG neurites in cell culture (Aletsee et al., 2001; our unpublished data). On homogenous LN surfaces, SG neurites showed a dose-dependent increase in length and number of neurites with increasing LN concentrations (Aletsee et al., 2001). However, in some areas of the inner ear, such as the osseus spiral lamina, ECMs appear to be present in more or less continuous surfaces (Woolf et al., 1992; Rodgers et al., 2001). In the organ of Corti, the target tissue for the SG dendrites, they appear to be present in stripes or tracks, and more than one ECM is present (Woolf et al., 1992; Cosgrove and Rodgers, 1997), suggesting that the differential composition of the ECM in the cochlea could provide guidance cues to SG dendrites. Alternatively, the ECM environment could alter the effect of a soluble factor on dendrite growth. Indeed, PLL or LN were found to alter dorsal root ganglion neurite reactions towards nerve growth factor and glial cell-line-derived neurotrophic factor (Tucker et al., 2006).

To explore the effects of a complex ECM environment on neurite growth in the cochlea, we evaluated the response of neonatal SG neurites to alternate stripes of ECM molecules. We hypothesized that neonatal SG neurites would respond to stripes of the ECM molecules when presented in alternation with the *in vitro* adhesion factor ploy-L-lysine (PLL) by turning onto the ECM stripe, extending on it and finally terminating on that stripe. Second, considering up to 550-μm distance between the SG and the organ of Corti, we hypothesized that the response to the ECM molecules would be dose-dependent, consistent with the concept of neurite targeting by gradients (Flanagan, 2006). Finally, we hypothesized that SG neurites would chose one ECM molecule, FN or LN, over the other when presented in direct stripe alteration.

METHODS

Preparation of Tissue Culture Plates

To prepare stripe molds, templates were generated by a photolithographic process to produce parallel stripes of photoresist compound, 100-μm wide, 100-μm high, and 100-μm apart. Silicone rubber (Sylgard®184 Silicone Elastomer, Dow Corning, Midland, MI) was then applied to the templates to generate stripe molds. The 4×7 mm^2 pieces of the resultant silicone molds were placed groove side down into 24-well cell culture plates (Costar®, Corning, Acton, MA), as shown in Figure 1(a), after sterilization with 100% ethanol for 20 min and air drying inside a sterile hood (The Baker Company, Sanford, ME). About 50 μL of the first ECM protein solution was applied to one side of the mold as shown in Figure 1(b). The ECM protein solution was drawn into the mold's channels by suction applied from the other side of the mold [Fig. 1(b)] and the plate was then incubated overnight at 4°C. Each well was then washed twice with phosphate buffered saline (PBS) with the molds in place as described above. In case of application of a second ECM protein, an additional blocking step with 2% bovine serum albumin (37°C for 2 h) was performed between the PBS washes. The molds were removed, followed by an additional wash with PBS [Fig. 1(c)]. Wells were then filled with 300 μL of PLL solution (5 μg/mL; Sigma-Aldrich, St. Louis, MO) or a second ECM protein solution and incubated at 37°C for 1 h. The wells were washed twice with Dulbecco's modified eagle medium (DMEM), (Gibco by Invitrogen, Carlsbad, CA) and filled with 170 μL of primary attachment

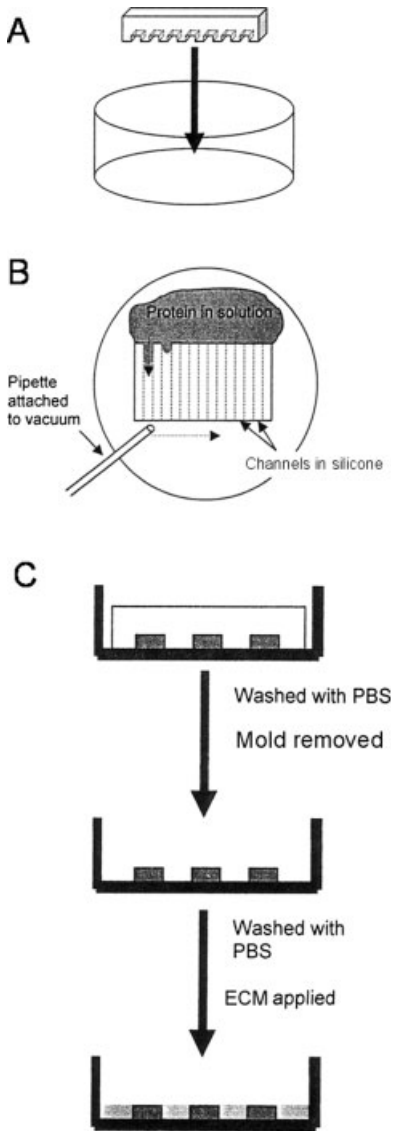

Figure 1 Preparation of ECM stripes for cell culture. (A) Placement of the mold into the cell culture well. (B) Application of ECM solution. (C) Washing and application of a PLL solution or second ECM solution.

medium, containing DMEM, 10% fetal bovine serum (Sigma-Aldrich, St. Louis,USA), 25 m*M* HEPES buffer (both Gibco by Invitrogen, Carlsbad, USA), and 300 U/mL Penicillin (Sigma-Aldrich, St. Louis, USA).

The ECM protein solutions for coating contained 5, 10, 20, 40, 80 mg/mL of either FN from human plasma, LN1 from Engelberth-Holms sarcoma (both Sigma-Aldrich, St. Louis, USA) or bovine serum albumin (BSA), (Chemicon, Temecula, CA) as a control protein. The conditions studied were (1) BSA stripes against PLL stripes, (2) LN stripes against PLL stripes, (3) FN stripes against PLL stripes, and (4) LN stripes against FN stripes.

Since some binding of a second ECM protein to stripes of the first ECM molecule could not be excluded after removal of the silicone matrix, experiments comparing LN versus FN were performed twice, with alternating coating sequence of the two ECM molecules.

Spiral Ganglion Dissection

The local animal subjects committee of the San Diego VA Health Care System approved the surgical procedures in accordance with the guidelines laid down by the National Institute of Health regarding the care and use of animals for experimental procedures. Neonatal (P5) Sprague–Dawley rats were deeply anesthetized by intraperitoneal injection of rodent cocktail (100 mg/mL ketamine, 100 mg/mL xylazine, 10 mg/mL acepromazine) 4 μL/g body weight before decapitation. The temporal bones were removed and further dissected similar to the method described by Van de Water and Ruben (1971). Briefly, the cochlear capsule was opened and the membranous labyrinth was removed from the modiolus. The spiral lamina containing the SG was carefully separated from the modiolus and transferred immediately into primary cell culture medium, where it was then cut into equal portions of 300–500 μm. Each portion (explant) was placed into an individual well of a culture plate, and at least 19 explants were studied under each experimental condition of stripe composition and ECM dosage.

Cell Culture

The explants first were incubated for 24 h at 37°C in the primary attachment medium, before the culture medium was changed to serum-free maintenance media (DMEM (Gibco), 25 m*M* Hepes-Buffer (Gibco), 6 mg/mL glucose (Gibco), 300 U/mL penicillin (Sigma-Aldrich), and 30 μL/mL N2-supplement (Gibco). For trophic support of SG neuron survival and optimization of neurite outgrowth in the stripe assays, maintenance medium was supplemented with 10 ng/mL of recombinant BDNF (R&D Systems, Minneapolis, MN). Cultures were kept in a humidified incubator at 5% CO_2 and 37°C for 72 h.

Immunohistochemistry

After fixation with 4% paraformaldehyde for 20 min at room temperature (RT) and two washes with PBS (Gibco), the explants were permeabilized with 5% Triton X-100 (Sigma-Aldrich) for 10 min, washed twice with PBS, and blocked for nonspecific antibody binding with 5% donkey serum (Sigma-Aldrich). Neurites were labeled for neurofilament using a polyclonal 200 kDa anti-neurofilament primary antibody (1:400; Sigma-Aldrich) while ECM stripes were visualized using polyclonal mouse or rabbit antibodies against LN, FN (both Sigma-Aldrich), or BSA (Fitzgerald Industries International, Concord, MA) at 1:400 each. After primary antibody incubation overnight at 4°C, followed by two PBS washes, the neurites and ECM stripes were visualized by 2.5 h of incubation with fluorescein isothiocyanate (FITC) or Texas-red (TR) conjugated secondary antibodies (1:100; Jackson Immunoresearch, West Grove, PA) against

the species of the respective primary antibody. Specificity of staining was confirmed by a series of negative control staining without primary antibodies.

Data Analysis

As noted above, at least 19 SG explants in stripe assays were evaluated for each experimental condition. (The few explants that failed to extend any neurites were excluded from analysis). Digital images were obtained on an inverted fluorescence microscope (Olympus IX70, Center Valley, PA) equipped with appropriate excitation and emission filters for FITC and TR. For publication in this manuscript, images were optimized to achieve uniform brightness and contrast using Adobe Photoshop (Adobe Systems, San Jose, CA).

The number of neurites terminating on each stripe substrate was counted for each individual explant. For each experimental condition, the neurite termination data of these explants showed normal distributions (in a Kolmogorow-Smirnow test) and homogenous variances. Therefore the individual explant data, grouped by experimental condition, were compared by ANOVA followed by Tukey post-hoc tests and paired t tests using Statistica 5.1 (StatSoft, Tulsa, OK). Results were considered to be significant when the likelihood for a Type 1 error was less then 5% ($p < 0.05$). Significance level was corrected according to the Bonferroni method when multipe tests were applied. The data presented in the text and figures are the means and standard deviations of individual explant data for each experimental condition. Although the PLL control concentration was constant at 5 μg/mL for all experiments, PLL data were treated as paired data with the respective protein stripe data, rather than as an isolated control group within the ANOVA, because it was assumed that the alternating ECM protein might influence the PLL data.

RESULTS

SG Neurites on Stripes of BSA Versus PLL

Before investigating the effects of the ECM proteins LN and FN, experiments were performed with BSA to control for possible mechanical or nonspecific protein boundary effects between stripes, due to the stripe generation process. As stated in the methods section, BSA was applied through the channels in the silicone matrix, which was then removed and the BSA stripes overlaid with PLL. This procedure involves the risk of a slight height difference between the stripes, possibly influencing neurite outgrowth, as well as a nonspecific protein difference. However, there was no significant difference between the number of neurites per explant ending on BSA compared with the number of neurites ending on PLL at 20, 40, or 80 μg/mL BSA (two-way ANOVA $p > 0.8$; Fig. 2). In addition, no obvious pursuit for growth along a particular type of stripe was detected, when the entire neurite was evaluated as opposed to the growth cone only [Fig. 3(a)]. Nevertheless, there was an overall effect of BSA coating concentration (two-way ANOVA $p = 0.001$; Fig. 2). The number of neurites terminating on PLL as well as on BSA decreased slightly with the increase in BSA coating concentration.

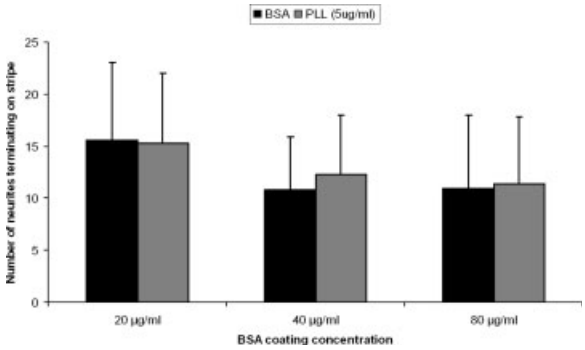

Figure 2 Equivalent termination of neonatal SG neurites on alternating stripes of BSA (20 to 80 μg/mL) versus PLL (5 μg/mL). Bars represent the mean number of neurites terminating on each substrate, per explant (error bar = 1 SD).

SG Neurites on Stripes of LN or FN Versus PLL

The growth behavior of neurites from neonatal SG neurons on stripes of LN opposed to PLL and stripes of FN versus PLL was not uniform, but dependent on the ECM molecule used in the experiment (LN–PLL, FN–PLL, three-way hierarchic ANOVA: $p < 0.0001$, Fig. 4). Neonatal SG neurons extended fewer neurites in the FN versus PLL experiment than in the LN versus PLL experiment. The ECM coating concentration used in the experiment had an overall effect on neurite outgrowth as well (three-way hierarchic ANOVA, concentration effect: $p < 0.021$). However, no over-all stripe effect of ECM molecule versus PLL was detected (three-way hierarchic ANOVA: $p > 0.05$), reflecting the fact that neurites showed different growth patterns for the different ECM molecules in the two PLL experimental series. While more neurites ended on PLL in the FN versus PLL experiments, more neurites ended on PLL than on LN at some concentrations and LN rather than PLL at others. This leads to two important interaction effects observed within the ANOVA. There was a strong interaction effect between the type of experiment (LN–PLL, FN–PLL) as well as the stripe type (ECM

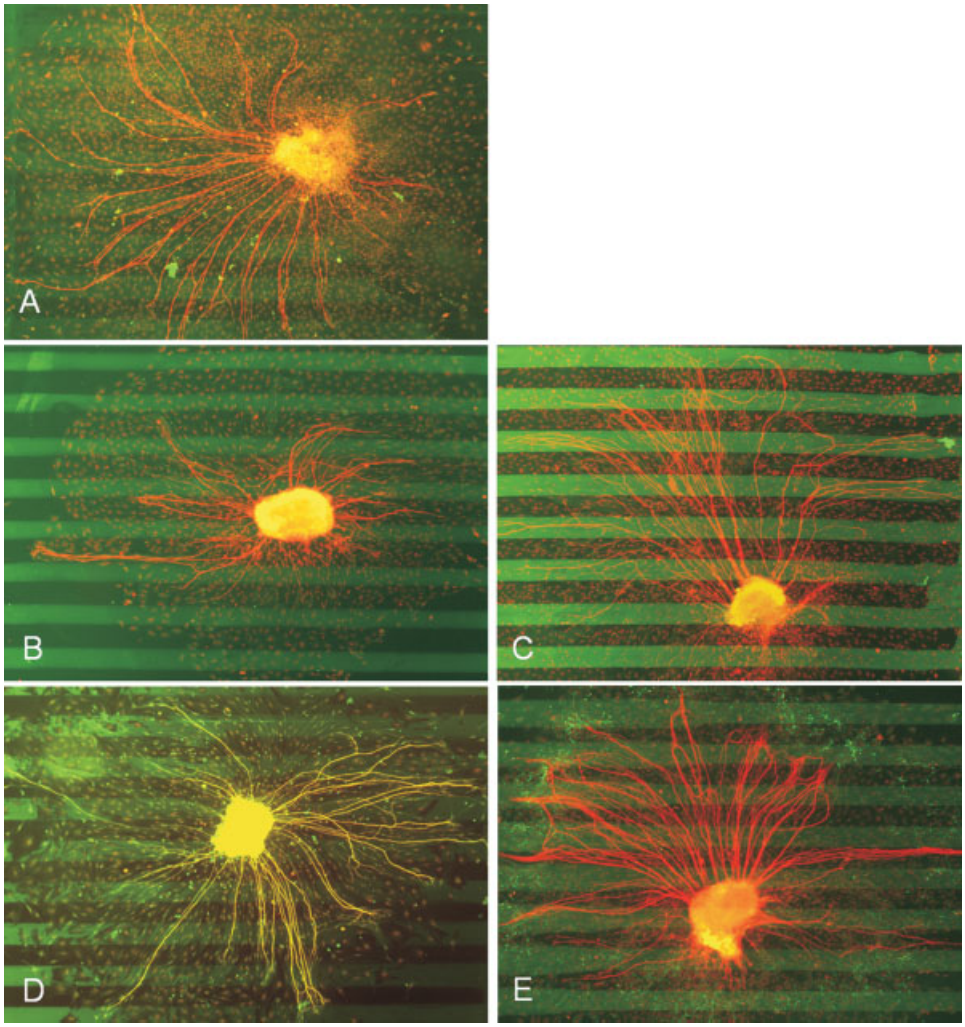

Figure 3 Representative examples for SG explants grown on various ECM stripe patterns after 72 h in culture. Stripe width = 100 μm. (A) Growth on BSA (80 μg/mL) versus PLL (5 μg/mL). There was no tendency for neurites to be found preferentially on either substrate. (B) Growth on LN (10 μg/mL) versus PLL (5 μg/mL). In this explant, neurites more often terminated upon and lay along PLL stripes. (C) Growth on LN (80 μg/mL) versus PLL (5 μg/mL). Neurites were preferentially observed on LN stripes. (D) Growth on FN (10 μg/mL) versus PLL (5 μg/mL). Neurites were distributed equally between FN and PLL stripes. (E) Growth on FN (80 μg/mL) versus PLL (5 μg/mL). Neurites were most often observed on PLL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or PLL) with the coating concentration of the respective ECM (ANOVA: $p < 0.004$ and $p < 0.0001$).

LN Versus PLL. At low LN concentrations (5 and 10 μg/mL), more neurites ended on PLL than on LN (post hoc paired t test, $p < 0.01$), while at the highest LN concentration of 80 μg/mL the observed trend reversed, with more neurites ending on LN than on PLL [post hoc paired t-test, $p < 0.001$, Fig. 4(b,c)]. In addition, neurites from many (but not all) explants clearly were observed lying along the PLL stripes (stripe ''tracking'') when PLL was opposed to 5 or 10 μg/mL LN stripes [Fig. 3(b)], while at 80 μg/mL extending neurites from many explants were clearly found along LN stripes [Fig. 3(c)]. At intermediate LN concentrations, examples of differential distribution on stripes were observed, but neurites from some explants were found on LN while those from others were found on PLL.

FN Versus PLL. Neurites extending from neonatal SG neurons on stripes of FN versus PLL preferentially terminated on PLL at 40 and 80 μg/mL FN (paired t-test: $p < 0.001$ and $p < 0.01$), while at lower

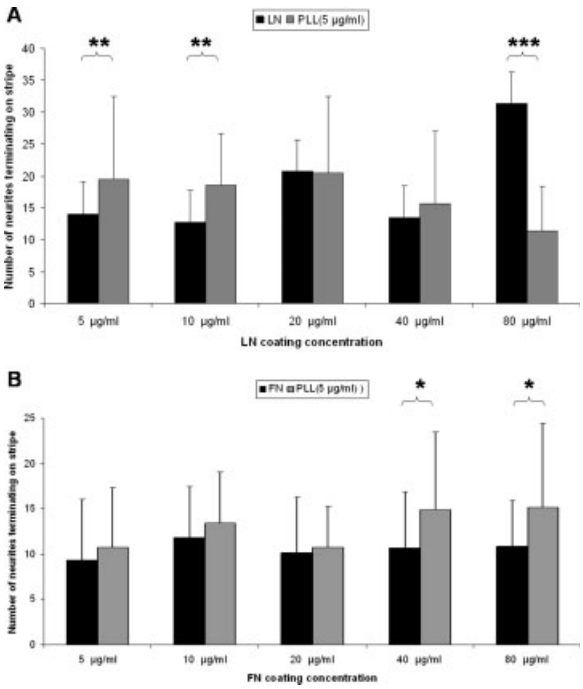

Figure 4 Dose-dependent, preferential termination of neonatal SG neurites on alternating stripes of (A) LN (5 to 80 μg/mL) or (B) FN (5 to 80 μg/mL) versus PLL (5 μg/mL); mean of neurites/explant and SD, post-hoc paired *t*-test, $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

FN concentrations, comparable numbers of neurites terminated on both substrates [Figs. 3(d,e) and 4]. In addition, at 40 and 80 μg/mL FN, for some explants the neurites clearly appeared to lie off of the FN stripes, and to be found along PLL stripes, along their lengths [Fig. 3(e)].

Stripe Restriction Depends Upon Initial Neurite Angle

As noted above, neurites were observed to lie along one type of stripe much of their length in many experiments. For the neurites showing such obvious stripe restriction, the initial angle of the neurite relative to the stripe border seemed to be of importance. When it occurred, stripe restriction was most prevalent for neurites in which the orientation of the initial portion of the neurite toward the stripes was at a shallow angle and least prevalent when the initial portion of the neurite was at right angles to the stripes (Figs. 3 and 5).

SG Neurites on Stripes of FN Versus LN

After investigating the effects of the individual ECM proteins LN and FN on SGN neurite extension by comparison against PLL-control, we then examined the effects of direct competition between LN and FN, by alternating stripes of the two ECM molecules, since the two ECM molecules occur in non-identical patterns in the perinatal organ of Corti (Woolf et al., 1992; Cosgrove and Rodgers, 1997; Rodgers et al., 2001). By immunofluoresence staining of the stripes, little deposition of the second ECM protein onto the stripes of the first ECM protein was observed (see Methods section). However, we repeated each experiment with the reverse sequence of ECM protein application, to control for any potential deposition or other effect of application order. ECM coating concentrations of 10 and 40 μg/mL were chosen for the competitive experiment, since in ECM/PLL assays neurites were found preferentially off of LN at 10 μg/mL but were not differentially distributed at 40 μg/mL, while FN showed no effect at 10 and neurites were observed more often off of FN at 40 μg/mL, when opposed to PLL. Based on these individual studies, one might then expect neurites to be observed off of LN at 10 μg/mL, and off of FN at 40 μg/mL.

Significantly more neurites terminated on LN than on FN in all experimental conditions, (Fig. 6). The coating concentration (10 and 40 μg/mL) did not influence this result. However, when LN was coated as the first stripe, increasing the coating concentrations from 10 to 40 μg/mL for both ECM proteins produced a stronger difference in neurite distribution (two-way ANOVA: $p < 0.0001$ for interaction between order and concentration).

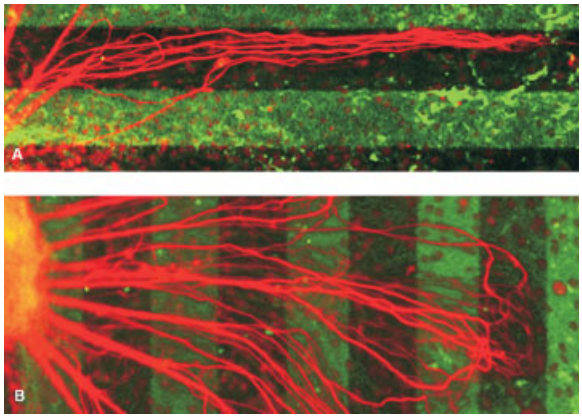

Figure 5 Stripe "tracking" by neurites was influenced by the angle at which the initial segment approached a stripe border: stripe tracking was most prevalent when the initial portion of the neurite was oriented at a shallow angle (A) and least prevalent when the initial portion was oriented head on (B). Stripe width 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

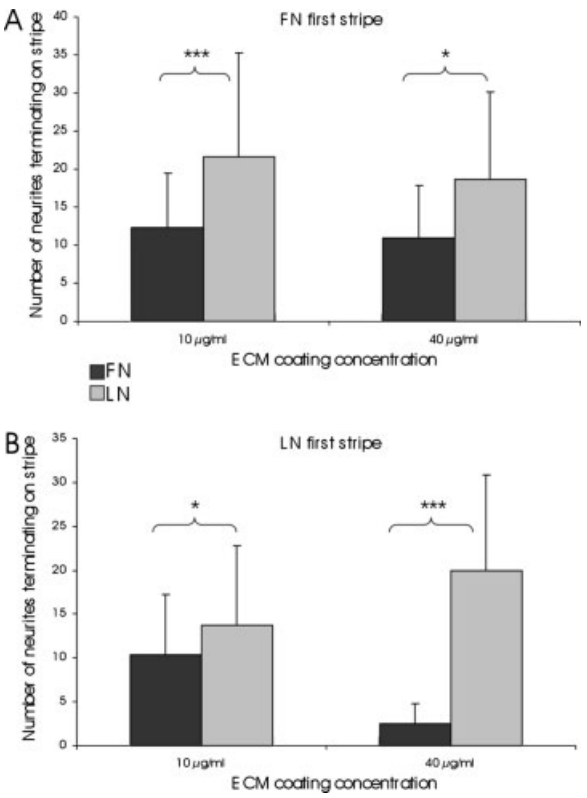

Figure 6 Termination of neonatal SG neurites on alternating stripes of LN versus FN with LN coated first (A) or FN coated first (B); mean and standard deviation, post-hoc paired *t*-test, $*p < 0.05$, $***p < 0.0001$.

DISCUSSION

The data presented in this study suggest that single or paired ECM molecules, presented in patterns, are sufficient to guide the neurites of neonatal SG neurons *in vitro*, indicating a potential role in dendrite guidance *in vivo*. We found that neonatal SG neurites respond to stripes of LN and FN when presented in alternation with the adhesion factor PLL. As hypothesized, the response to both ECM molecules was dose-dependent. In the LN versus PLL assay, neurites were preferentially found off of LN at low concentrations (5 and 10 μg/mL) and preferentially on LN at high concentration (80 μg/mL). In the FN versus PLL assay, neurites were most often found off of FN stripes, but only at high FN coating concentrations (40 and 80 μg/mL). In the direct competition between FN and LN, neurites were found preferentially on LN at low (10 μg/mL) as well as at high (40 μg/mL) concentrations. These results suggest that SG neurites actively preferred or avoided certain substrates, although in the absence of kinetic information we report only the final positions of the neurites.

Stripe Assays to Investigate Functional Guidance Cues in SG Neurite Outgrowth

Previous studies have used stripe patterns of LN to investigate *in vitro* neurite guidance in various neuron populations (Hammerback et al., 1985; Vielmetter et al., 1990; Gomez and Letourneau, 1994). In the later two studies, the stripe generation methods described were virtually identical to those used in the present study, using a mold system to apply the first ECM protein and then applying the second ECM to the entire dish after mold removal and blocking with hemoglobin or BSA (Gomez and Letourneau, 1994). The molds generated stripes with clear borders. We detected minimal binding of the second applied ECM on the surface coated with the first ECM molecule by immunofluorescence (data not shown). Concordantly, Gomez and Letourneau (1994) detected less than 6% binding of the second ECM molecule to the surface coated with the first ECM at ECM concentrations of 50 μg/mL. However, we repeated competition experiments of LN versus FN with alternate coating sequences, and observed the same results (see Fig. 6), suggesting that limited overcoating by the second ECM molecule was not confounding the results.

In our stripe assay experiments we used organotypic explants of the inner ear's SG. Besides neurons, the explants contained supporting cells, including fibroblasts and Schwann cells, which reside between the neurons *in vivo*. Both cell types are known to provide guidance cues to advancing neurites and growth cones, and therefore might have influenced the observed neurite patterns in our experiments. However, these supporting cells grew out in a homogenous, radial way from the explants (see Fig. 3). We did not see a clear alignment of cells to either FN or LN stripes, as Thompson and Buettner (2006) reported for LN in neonatal rat sciatic nerve Schwann cells. This suggests that the response of SG neurites to ECM patterns may have been mediated directly, rather than via Schwann cells or fibroblasts, although this possibility must still be considered.

SG Neurites Respond to ECM Stripes in a Dose-Dependent Manner When Opposed to PLL

In the FN versus PLL assay, neurites appeared to avoid FN stripes at high FN coating concentrations (40 and 80 μg/mL). This threshold effect suggests that FN would have to be present in relatively high concentrations to influence SG neurite projections *in vivo*. Cytochemical localization in the developing organ of Corti, while not quantitative, shows intense

labeling around the dendrites of the SG neurons, and in tracks beneath the HCs of the organ of Corti (Woolf et al., 1992). This suggests that FN is present in relatively high concentrations during the late stages of sensory epithelial innervation, when neurites are remodeling and when initial synapses are forming with the HCs (e.g. Hafidi et al., 1990; Pujol et al., 1998). While it has been speculated that FN may serve as a substrate for directing neurite growth in the developing organ of Corti (Woolf et al., 1992), our observation of apparent FN avoidance suggests that this ECM molecules may function to channel neurites toward their sensory cell targets. Alternatively, FN may serve to reduce the projection rate of SG dendrites as they near their HC targets. Kuhn et al. (1995) has shown that chick dorsal root ganglion (DRG) neurites slow down as they approach a bead coated with FN. FN beneath the HCs could thus serve as a stop signal for projecting SG dendrites, giving the growth cone filopodia more time to explore the region and to find their HC targets.

In the LN versus PLL assay, neurites appeared to avoid LN at low concentrations (5 and 10 μg/mL) while they apparently preferred LN at a high concentration (80 μg/mL). This suggests that SG neurites can respond to lower levels of LN in comparison with FN. Moreover, the differential response across concentration suggests that SG neurites could respond to a gradient of LN, by turning in the direction of increasing concentration. Currently there is no evidence for LN gradients in the developing cochlea. However, a decreased concentration of LN could serve to slow or terminate SG neurite projection, since Aletsee et al. (2001) have indirectly shown that the rate of SG neurite extension on LN is dose-dependent. In their experiments, neurite length observed after 72 h in cell culture increased with increasing LN concentrations. Additionally, Dolda and Bellamkonda (2006) recently found that neurites of chicken DRG respond to anisotrophic LN-1 scaffolds with higher extension rates than to homogenous LN-1. In the inner ear, Rodgers et al. (2001) detected strong immunoreactivity for LNs in the projection path of developing SG dendrites through the osseus spiral lamina and basal lamina of the organ of Corti, but only weak labeling in the organ itself.

SG Neurites Appear to Prefer LN Over FN

When stripes of LN and FN directly opposed each other, SG neurites apparently preferred LN over FN at two coating concentrations (10 and 40 μg/mL). These results are consistent with Vielmetter et al.'s (1990) observation in goldfish retinal axons, with axons preferentially extending on LN (20 μg/mL) than on FN (50 μg/mL). They found LN to be the most permissive substrate for neurite outgrowth amongst several tested substrates. Also, Kuhn et al. (1995) observed that growth cones of chick DRG fibers extending on FN change their direction towards LN-coated beats.

Stripe "Tracking" of Neurites

We often observed stripe ''tracking'' behavior, with neurites apparently following PLL stripes in opposition to high concentrations of FN and low concentrations of LN, and following LN at high concentrations (Figs. 3 and 5). This stripe tracking appeared to depend upon the angle at which neurites approached the stripes, with a higher probability of tracking if the approach was made at a shallow angle. This could reflect the signal presented to the growth cone when approaching a stripe. At a perpendicular angle of approach, filopodia could deliver symmetrical signals to the growth cone, resulting in stripe crossing. At a shallow angle, the signal received by the growth cone could be highly asymmetric, resulting in turning to maximize the preferred signal. It should be noted that we did not see any evidence of sharp turns near borders in most neurites. Of course, this could reflect remodeling of the neurite after the crossing. Indeed, using live videomicroscopy, Gomez and Letourneau (1994) noted that the angle of DRG neurite approaching to a LN/FN stripe border measured in post-fixation imaging did not reliably reflect the actual angle at which the neurite's growth cone had encountered the border.

Even when approach angle was taken into consideration, the stripe tracking of neurites observed in this study was not necessarily homogenous for all neurites extending from one explant, or from different explants at one concentration of an ECM molecule. Especially in experiments with intermediate LN concentrations opposed to PLL, neurites from a single explant showed PLL tracking alongside LN tracking, or different explants in the same experimental condition showed opposite tracking preferences. These data suggest that SG neurons are a heterogeneous population. Indeed, there are two subtypes of SG neurons, the Type I and Type II SG neurons (e.g. Reid et al., 2004), which have different target cells and functions. Type I cells form afferent synapses with the inner HCs, while Type II cells form afferent synapses with the outer HCs in the organ of Corti (Reid et al., 2004). However, less than 5% of neurons in the

SG are Type II neurons, and we did not see any subpopulation that fit this proportion.

Another factor potentially influencing the likelihood of stripe tracking might be the initial orientation of the explant to the stripes. SG explants are often ''polar'' in organotypic cell culture, with neurites preferably growing out of one side of the explant [see Fig. 3(e,c)]. We did not control for the orientation of the explant towards the stripes. Thus variable positioning of explants within one experimental condition might have contributed to the inhomogenous tracking behavior of neurites from different explants in this group. However, as orientation was to our knowledge random for all explants, it should not have biased tracking toward any particular striped molecule.

Finally, another explanation for the somewhat inhomogeneous response of neurites from different explants could involve the origin of the explants along the length of the cochlea. The neurons in the different turns of the cochlea are known to vary in their characteristics, including their response to growth factors (e.g. Davis, 2003). Since we used explants from throughout the cochlea in our experiments, different turn origin of the explants might account for variable tracking behavior of different explants.

CONCLUSIONS

Our results are consistent with the idea that patterns of LN and FN help to direct SG neurite outgrowth during the innervation of the inner ear, as has been proposed at other sites (e.g. Woolf et al., 1992; Rodgers et al., 2001). Moreover, our observations suggest a potential model for neurite guidance. High concentrations of LN in the SG and osseus spiral lamina may promote SG dendrite growth through this structure and toward the organ of Corti. Within the organ of Corti, lower concentrations of LN may provide a reduced signal and slow neurite growth. FN beneath each row of HCs may provide a stronger stop or avoidance signal. This could allow growth cone filopodia increased time to sample their cellular targets, or might even direct the fibers upward toward the HCs. Gradients of LN could also provide directional signals.

Further studies focusing on the ECM microenvironment and receptor distribution within the organ of Corti are needed to elucidate the impact of the ECM on neurite guidance and synapse formation. Moreover, the sensory epithelium of the cochlea is a three-dimensional structure. Patterning of substrates in 3D has been used by many groups to extend observations obtained in 2D (e.g. Yu et al., 1999) and could be applied to SG neurons as well. In addition, ECM molecules act in concert with other guidance cues including soluble growth factors (Ryan et al., 2006). Evaluating neurite responses to combined cues will be required to more fully understand developmental events in the inner ear.

The authors thank Dr. Richard Lieber, University of California San Diego, Department of Orthopedic Surgery, for advice on statistics. We thank Kwang Pak, University of California San Diego, Department of Surgery/Otolaryngology, for excellent technical assistance.

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