Published in final edited form as:

Biomaterials. 2012 June ; 33(17): 4288–4297. doi:10.1016/j.biomaterials.2012.02.046.

The role of well-defined patterned substrata on the regeneration of DRG neuron pathfinding and integrin expression dynamics using chondroitin sulfate proteoglycans

Gerald N. Hodgkinson, Patrick A. Tresco, and Vladimir Hlady*

Department of Bioengineering, University of Utah, Salt Lake City, Utah 84112

Abstract

Injured neurons intrinsically adapt to and partially overcome inhibitory proteoglycan expression in the central nervous system by upregulating integrin expression. It remains unclear however, to what extent varying proteoglycan concentrations influence the strength of this response, how rapidly neurons adapt to proteoglycans, and how pathfinding dynamics are altered over time as integrin expression is modulated in response to proteoglycan signals. To investigate these quandaries, we created well-defined substrata in which postnatal DRG neuron pathfinding dynamics and growth cone integrin expression were interrogated as a function of proteoglycan substrata density. DRGs responded by upregulating integrin expression in a proteoglycan dose dependent fashion and exhibited robust outgrowth over all proteoglycan densities at initial time frames. However, after prolonged proteoglycan exposure, neurons exhibited decreasing velocities associated with increasing proteoglycan densities, while neurons growing on low proteoglycan levels exhibited robust outgrowth at all time points. Additionally, DRG outgrowth over proteoglycan density step boundaries, and a brief $\beta 1$ integrin functional block proved that regeneration was integrin dependent and that DRGs exhibit delayed slowing and loss in persistence after even transient encounters with dense proteoglycan boundaries. These findings demonstrate the complexity of proteoglycan regulation on integrin expression and regenerative pathfinding.

1. Introduction

Regenerating neurons possess the ability to modulate integrin expression, enabling navigation of various extracellular environments after injury. Notably, elevated integrin expression has been associated with axotomy [1], and in *in vitro* experiments integrin upregulation is sufficient in some cases to overcome the inhibitory effects of chondroitin sulfate proteoglycans (CSPGs) [2,3], the major inhibitory constituent in central nervous system (CNS) injuries [4,5]. In recent years, many promising CNS repair strategies have focused both on diminishing inhibitory signals at the site of injury and augmenting the intrinsic ability of neurons to extend new processes and to find a path through injured tissue [6–12].

© 2012 Elsevier Ltd. All rights reserved

*Correspondence to: Vladimir Hlady 20 S. 2030 E., Rm 108A Department of Bioengineering, University of Utah, Salt Lake City, Utah 84112 ph. 801-581-5042 fax 801-585-5151 vladimir.hlady@utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Neurons such as dorsal root ganglia (DRG) have been studied for their ability to regenerate after spinal cord injuries and have served as a model cell type for many CNS injury studies. In vitro and in vivo studies have revealed that DRGs, regardless of age, require a number of conditions for robust regeneration including 1) sufficient growth promoting ECM molecules such as laminin for anchor dependent locomotion [13,14], 2) growth factors such as NGF or NTF [15–17], 3) expression of active ECM anchoring receptors such as integrins [3,18–19], and 4) active expression of second messenger systems such as cAMP [20,21]. DRGs, unlike non-neuronal migrating cell types, possess the capacity to adapt to a wide range of substrate adhesivities such that robust pathfinding can proceed on diffuse concentrations of laminin or even in the presence of varying concentrations of inhibitory proteoglycans [18,22–24]. Various means have also been devised to experimentally increase DRG outgrowth through inhibitory boundaries, including by increasing integrin expression by viral transfection [2], activating existing integrins into an ECM binding conformation [20,22], increasing cAMP intracellular levels [20,21], providing growth factors [15–17], and by removing activity of a recently discovered receptor for CSPGs [25].

Despite of in vitro evidence that neurons adapt to CSPG signals, there are uninvestigated aspects of this phenomenon which, if better understood, could provide insights into more effective harnessing the intrinsic regenerative capacity of neurons. For instance, there is little to no time-resolved data on integrin expression and pathfinding dynamics of neurons navigating environments of varying proteoglycan densities. Additionally, integrin expression data has most often been collected from pools of neurons, yielding an average population response that masks heterogeneity between individual neurons, and which samples integrin expression in cell structures not contributing to the pathfinding response. Because of these shortcomings in the literature it is unclear to what extent neurons modulate growth cone integrin expression and pathfinding behavior in response to various CSPGs densities, how quickly neurons adapt integrin expression to CSPG signals, or what concentration of CSPG represents an upper limit beyond which neurons cannot intrinsically adapt.

To better elucidate the effects of CSPG signal strength and duration on DRG adaptation and regeneration, we developed in vitro substrata that allow the direct interrogation of growth cone integrin expression and pathfinding dynamics on varying uniform CSPG substratum densities and concentration step boundaries. These substrata enabled us to precisely control CSPG spatial presentation and to deduce the influence of local CSPG levels on pathfinding velocity and persistence. By controlling CSPG density we could simulate the effects of DRGs growing into a CSPG barrier at a CNS injury site or across and out of a CSPG rich zones, thus facilitating a better understanding of the intrinsic ability of DRGs to overcome these barriers to regeneration.

2. Materials and methods

2.1. Micropattern device fabrication

Micropatterning devices were designed, modeled, and fabricated using facilities of the University of Utah Microfabrication Laboratory. Patterns were modeled in Autocad R14 (Autodesk), translated onto photolithographic masks (Nanofilm) using a pattern generator and image repeater (Electromask) at 5× magnification, then developed and etched to produce final masks through 5× reduction exposure. Resulting masks were prepared as microfabrication molds by developing, etching, then curing at 160°C for 30 minutes to stabilize the patterned photoresist layer. Pre-polymer polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) was poured onto molds and placed under vacuum to ensure filling of pattern microwells. Stamps were cured at 100°C for 90 minutes then removed, soaked

overnight in hexanes (Aldrich), and dried by heating to remove solvents and low molecular weight polymerization residues.

2.2. Protein preparation and dye conjugation

Laminin (LN) isolated from mouse Englebreth-Holm-Swarm sarcoma (Invitrogen) and aggrecan (AG) isolated from bovine articular cartilage (Sigma) were conjugated with Alexa Fluor 488 succinimidyl ester dye (AF488, Invitrogen) using protocols suggested by Invitrogen. Alternatively, Alexa Fluor 594 succinimidyl ester dye (AF594, Invitrogen) was mixed with aggrecan in a 10:1 AF594:AG molar ratio to produce a physically labeled AG species. All covalently labeled proteins were isolated through gel permeation chromatography using Sephadex G-25 columns (GE Healthcare). Free dye removal from the labeling reactants was verified with thin layer chromatography. Final protein solution concentrations and dye to protein molar ratios were determined through absorbance spectroscopy and the Bradford method using UV/Vis spectrophotometry.

2.3. Surface patterning

Glass coverslips were cleaned prior to surface modification in a 1 H₂O₂: 2 H₂SO₄ mixture, rinsed 4× with double distilled deionized (DD) water, then dried under a nitrogen stream. PDMS devices were cleaned with ethanol then dried prior to protein adsorption. Laminin (500 µg/ml) was adsorbed on glass coverslips for 1 hour to produce an average uniform coverage of 334±53 ng/cm² (± stdev) and aggrecan (1 mg/ml) was applied to PDMS devices for 20 minutes. After protein adsorption, glass coverslips and PDMS stamps were rinsed with DD water then briefly dried under a nitrogen stream. In a microcontact printing (µCP) process, AG coated PDMS stamps were brought into conformal contact with LN coated coverslips to transfer the adsorbed AG layer. Stamps were designed to generate a 1×1 cm² pattern of six repeating, parallel 30 µm wide LN outgrowth lanes, containing either only LN or AG clusters at 2, 4, 7, 11, & 31% average densities. Lanes were separated by uniform 100 µm wide AG coated fields (100%). Negative controls consisted of uniformly LN coated substrates without AG patterns.

Additional stamps were designed to generate a 1×1 cm² pattern of five repeating, parallel 30 µm wide outgrowth lanes consisting of a LN only lane, a 11% AG lane, and three lanes each containing a different combination of alternating low and high AG densities (LN Lane to 11%, 3 to 11%, and 5 to 11% AG). In these alternating step density lanes, LN only and low density AG sections were 500 µm in length and 11% density sections were 250 µm in length.

2.4. Protein pattern density determination

A fluorescence microscopy imaging technique described in previous work [26] was utilized to determine the surface density of protein layer or clusters. Laminin densities were determined from three separate samples imaged at 8 to 10 locations each. Three separate samples of each AG pattern were produced on a homogeneous, non-conjugated LN film and imaged at 8 to 10 locations for each distinct AG density in each sample. Average AG density for each distinct cluster pattern was calculated by dividing the protein content in 10 clusters by the corresponding lane area. Imaging of fluorescently labeled LN films revealed that AG patterning did not alter LN film densities. Image Pro v4.1 software was used to process images and quantify fluorescence intensities.

2.5. DRG isolation and culture

All procedures involving animals were conducted using sterile technique in accordance with protocols approved by the National Institutes of Health Guide for the Care and Use of

Laboratory Animals and the University of Utah Institutional Animal Care and Use Committee. Postnatal 1–2 day Fisher rat DRG neurons were explanted, cleaned, and purified. Ganglion bodies were taken from all accessible levels of the vertebral column then placed in Leigovitz's L-15 medium (Invitrogen) and stripped of dorsal roots and connective tissue. Ganglia were dissociated with 1.33% collagenase (Sigma) for 30 minutes then with 0.25% trypsin for 15 minutes, followed by trituration through a fire polished Pasteur pipette in 0.1% DNase (Worthington) and soybean trypsin inhibitor. Contaminating Schwann cells were killed by the addition of 20 μ l purified O4 antibodies to a 1 ml cell suspension in DMEM then by the addition of 50 μ l purified rabbit complement. Cell supernatants were resuspended in serum free SATO⁻ medium [29] containing 10% DMSO and placed in liquid nitrogen storage for later use.

All patterned substrates were treated with 500 μ g/ml gentamicin (Sigma) in sterile PBS for 15 to 30 minutes then rinsed with PBS 3 $\times$ prior to cell seeding. Cells were quickly thawed, removed from DMSO by dilution and centrifugation, then resuspended in SATO⁻ cell culture medium + 10 ng/ml NGF. DRGs were seeded at a density of 1.5k viable cells/sample.

2.6. Growth cone pathfinding experiments

2.6.1. Phase time lapse video microscopy—Time lapse video microscopy experiments were conducted on a Nikon Diaphot 200 inverted microscope equipped with a sample cell holder and Biopetechs sample heater, a Princeton Instruments CCD camera, and an automated microscope stage controlled by OpenLab v3.1.5 software. A Nikon 10 $\times$ Phase 1 objective was used for both phase and fluorescence imaging. With this system, up to 40 different locations were monitored for growth cone movements in one sample. DRGs were seeded on protein patterns or on laminin control surfaces deposited on the glass bottom of Delta T dishes (Biopetechs). DRGs were allowed at least 2 hours in an incubator for substratum attachment before T dish placement on the heated microscope stage. Locations containing attached viable DRGs were selected at random and imaged over time with phase optics. Initial fluorescence images of the AF594 labeled pattern were captured and overlaid on phase images to track cell and growth cone location in relation to the pattern. Composite images were placed in sequence and growth cone positions vs. outgrowth time were measured using Image J v1.31 software.

2.6.2. Pathfinding parameters and analysis—Growth cone dynamics were measured and analyzed for the following parameters: 1) velocity per time lapse increment, 2) straight line distance per a given time period of outgrowth, or 3) persistence, which was defined as a percentage of movements contributing to straight line distance per a given time period of outgrowth. Only single dissociated DRG neurons were measured for outgrowth, and measurements were not continued on growth cones that had encountered debris or other cell structures. Growth cone position at each time point was measured as the center of the leading edge of the growth cone. Leading edge filopodia were not in measurements unless by morphological evidence filopodia had transformed into a growth cone structure. Pathfinding dynamics were captured every 15 min for 15 hr duration experiments and every 5 min for AG density step function and integrin block experiments.

For continuous AG density lanes, pathfinding data was collected from two experiments on AG patterns and from one experiment on non-patterned LN controls. Outgrowth dynamics were monitored from 3 to 15 hours post seeding and were divided into four 3 hr time blocks for analysis. For Step boundary AG density lanes, pathfinding data was collected in three separate experiments beginning at 3 hours post seeding and for up to 3 hours post step boundary crossing. Data was time-aligned by AG boundary crossing event. Growth cone

dynamics were calculated on an hourly basis to determine rapid changes caused by boundary crossing.

2.6.3. Integrin functional block assay—The ability of hamster anti-rat CD29 β 1 integrin receptor antibody (α CD29, BD Biosciences) to block DRG neurite extension in SATO⁻ + NGF media on a homogeneous LN field was determined in a time and antibody concentration titration experiment (data not shown). A minimal α CD29 concentration was chosen that blocked neurite outgrowth for at least up to 2 hours after administration. DRGs seeded on patterns containing differing continuous AG densities were pulsed for 1 hr with α CD29, starting at 5 hours post seeding, by injecting 40 μ l of 1 mg/ml α CD29 into the Delta T dish to produce a final concentration of 20 μ g/ml. At the end of 1 hour the growth media was gently exchanged 3 $\times$ to remove the blocking antibody. Growth cone movements on various AG densities were imaged before, during, and after integrin block in two separate experiments, and in one experiment on a non-patterned LN control sample. Cells were monitored for growth cone retraction and cell body detachment from the substrate. Only growth cones that recovered from the block were included in pathfinding calculations. Dynamics were calculated separately for the first half and second half of integrin block then on an hourly basis thereafter to monitor rapid changes due to changing blocking conditions and functional integrin recovery.

2.7. DRG receptor staining

DRGs seeded on the various AG density patterns were fixed at 6, 9, 12, and 15 hours post seeding for cell staining experiments. Two samples at each time point were stained for α 1 β 1 or α 6 β 1 integrins. A blocking solution of 4% goat serum + 0.3% Triton in PBS was used to dilute all antibodies and to permeabilize cells prior to primary antibody incubation. Cells were incubated in mouse anti-rat α 1 β 1 integrin receptor IgG2a (10 μ g/ml, Millipore) at room temperature for 1 hour, rinsed 3 $\times$ with PBS, rinsed once with blocking solution, incubated for 1 hour in goat anti-mouse IgG (H&L) AF488-antibody (1:500 v/v Invitrogen). For α 6 β 1 labeling, cells were incubated in mouse anti-rat α 6 β 1 integrin receptor IgG1 (1:200 v/v, Millipore) [28] at room temperature for 1 hour, rinsed 3 $\times$ with PBS, rinsed once with blocking solution, then incubated for 1 hour in goat anti-mouse IgG1 AF488-antibody (1:500 v/v, Invitrogen).

Images of labeled integrin receptors in intact growth cones were captured using either a Nikon Plan Apo, 60 $\times$, 1.4 N.A. or Nikon Plan Fluor, 100 $\times$, 1.3 N.A. objective and processed by correcting for uneven illumination and subtracting background noise levels. Image Pro v. 1.4 and MS Excel were used to calculate total fluorescence intensity and intensity per growth cone area. Expression densities were expressed as ratios to control neuron integrin expression on non-patterned LN substrata at 6 hours post seeding.

2.8. Statistical

All statistical analyses were performed with SPSS v12.0 software. An ANOVA one-way analysis was conducted on groups to determine $p < 0.05$ significance before making multiple post-hoc t-test comparisons. T-test p values were two-tailed and found assuming unequal variance. In the case of non-parametric data, a Kruskal Wallis (KW) analysis was conducted on groups to determine $p < 0.05$ significance before making multiple post-hoc comparisons using a two-tailed Mann Whitney (MW) test.

3. Results

3.1. Growth permissive lanes with continuous CSPG densities

Microfabrication techniques were utilized to produce precise CSPG patterns superimposed on a uniform laminin (LN) (334 ± 53 ng/cm²) background through μ CP of the CSPG aggrecan. Patterns contained six repeating 30 μ m wide parallel lanes each separated by 100 μ m wide fields of AG and with lanes containing either only LN or a unique AG density of patterned inhibitory clusters, each consisting of 1 – 3 AG molecules (Fig 1A&B). Table 1 lists the average AG density per lane as calculated on a percent of maximal AG density found between lanes, designated as 100%.

3.2. DRG outgrowth dynamics vs. AG density

DRG growth cone pathfinding dynamics were monitored through time lapse video microscopy starting at 3 hr post seeding and by taking images every 15 min over a 15 hr time period to elucidate changes in pathfinding behavior induced by CSPG signals. DRGs adhered to all outgrowth lanes with no apparent preference for lower AG densities and extended growth cones onto all AG densities including onto 100% AG fields located between lanes (Fig 1C). As seen in similar experiments [29,30], outgrowth was generally constrained to outgrowth lanes. Neurons in all AG density groups were seen to contact AG boundaries at least at initial time points by lamellipodia and filopodia sampling.

Control neurons on non-patterned LN substrates maintained a consistent average outgrowth velocity throughout the 15 hr outgrowth period, neurons on AG patterns exhibited widely different responses depending on beginning at 42.6 ± 2.0 μ m/hr, while lane AG density (Fig 2A). Several AG densities promoted significantly faster outgrowth velocities versus controls during the first two time frames (2%, 7%, 11% & 31% AG), while outgrowth slowing proceeded in an AG dose dependent fashion by the end of the fourth time frame, with 100% AG neurons slowing most on average. Notably, neurons in LN only lanes also significantly slowed over time, unlike neurons growing in similar conditions on 2% and 4% AG lanes, which did not slow compared to controls at any time point.

Outgrowth persistence was measured as a function of AG density and outgrowth time to investigate possible changes in outgrowth trajectory. Neurons growing on lanes containing only LN or 2% thru 11% AG densities exhibited significantly greater persistence in a single outgrowth direction compared to controls, while outgrowth on the higher AG densities of 31% and 100% was statistically the same as controls (Fig 2C). Despite of a tendency in slowing velocities, all neuron groups, with the exception of neurons on LN only lanes, did not vary significantly in persistence over time. Specifically, neurons on LN only lanes exhibited significantly greater persistence than all other groups in the first time frame, but significantly less than three other neuron groups (2%, 7%, & 11% AG) by the third time frame.

3.3. $\alpha 1\beta 1$ and $\alpha 6\beta 1$ growth cone expression vs. AG density

To investigate the effect of AG substratum density on integrin expression dynamics during regeneration and pathfinding, we imaged intact growth cones on all substrata to interrogate $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin expression on a 3 hr resolution time scale (Fig 3). $\alpha 1\beta 1$ expression patterns were mostly unaffected by AG substratum density (Fig 3A), although neurons growing on LN only lanes, 7% AG, or 31% AG lanes significantly upregulated $\alpha 1\beta 1$ expression compared to controls at 9 hr post seeding. Also of note were neurons on 7%, 31%, and 100% AG densities, which expressed higher levels of $\alpha 1\beta 1$ integrin than neurons on LN only lanes by the final time frame, indicating perhaps a slight AG dose dependence in integrin upregulation over time.

$\alpha 6 \beta 1$ modulation in response to AG was pronounced, with all neuron groups exhibiting upregulation for at least one time point, and with peak integrin expression in 100% AG neurons reaching nearly 2.5 $\times$ that of controls at 6 hr (Fig 3C). In general, $\alpha 6 \beta 1$ was AG dose dependent as evidenced by the number of time points for which 100% AG neurons expressed significantly higher levels of $\alpha 6 \beta 1$ than other groups. Interestingly, most neuron groups, including controls, exhibited transient peak $\alpha 6 \beta 1$ expression at 9 hr, indicating perhaps a response to axotomy, which has also been observed in DRGs in vivo [1].

3.4. Effect of integrin upregulation on outgrowth velocity

In order to deduce possible effects of integrin upregulation on DRG outgrowth dynamics we plotted DRG velocity per $\alpha 6 \beta 1$ growth cone density as a function of AG density and outgrowth time (Fig 3E). In general, controls exhibited higher velocity to integrin density ratios than neurons growing on AG patterned lanes, indicating a lower requirement for total integrin content to maintain outgrowth velocities in the absence of AG signals. Neurons on AG patterns followed a general trend of decreasing velocity to integrin expression ratios with increasing AG density, indicating diminished translation of integrin content to outgrowth velocity, even at early time points. Importantly, neurons growing on LN only lanes exhibited velocity to integrin ratios less than that observed for controls or for 2% and 4% AG neurons, despite the lack of a pronounced AG signal in outgrowth pathways for any of these groups.

3.5. Outgrowth dynamics during and after anti- $\beta 1$ integrin block pulse vs. AG density

To determine the contribution of observed integrin expression upregulation on outgrowth dynamics we assayed neuronal outgrowth during and after $\beta 1$ integrin block employing an antibody we found to completely inhibit DRG neurite outgrowth on non-patterned LN substrata (see Methods Section). Outgrowth in control neurons stopped or retracted on average during blocking conditions, while neurons on AG patterns were less affected. Only 47% of control neurons recovered from the block, with 18% being washed off during solution exchange, while all neurons on 31% and 100% AG densities remained attached, and recovered from the block (Fig 4A). Additionally, control neurites contracted the most immediately upon block initiation, while most AG pattern neuron groups exhibited average positive outgrowth while still in the presence of the blocking antibody (Fig 4B), indicating AG exposure induced an ability to overcome blocking conditions before antibody removal. Additionally, neurons on LN only lanes and on 2% and 4% AG lanes all exhibited significantly greater persistence in outgrowth than controls during the integrin block (Fig 4C).

Once blocking antibodies were removed, several neuron groups, including controls, responded immediately by improving persistent outgrowth, indicating rapid recycling of membrane expressed integrins in active LN binding conformation. Post block outgrowth indicated a biphasic response in which neurons growing on low levels of 2% thru 7% AG densities recovered most robustly from the block, while neurons on greater AG densities tended to slow or stop altogether by the end of the 3 hr recovery (Fig 4C). Neurons growing on 2% AG exhibited robust straight line outgrowth and the most pronounced recovery terms by the end of 3 hr in terms of persistent outgrowth, indicating reserve integrin pools in these neurons were less affected by blocking conditions (Fig 4C).

3.6. Outgrowth dynamics on AG density step boundaries

To more fully investigate the effects of AG conditioning on DRG outgrowth into higher or lower AG densities, we created a second set of patterns in which outgrowth lanes contained step boundaries of various AG densities (Fig 5A, Table 2). In general, outgrowth behavior was dependant on the magnitude of the step boundary being crossed. Neurons did not

significantly change velocity when crossing from 3% to 11% AG or from 11% to 3% AG or from 11% to 5% AG, and only slightly changed velocity when crossing from 5% to 11% AG, suggesting intermediate changes in AG density had little short-term effect on DRG outgrowth velocity (Fig 5B). Conversely, DRGs crossing from high to low AG densities of 100% to a LN only lane or from 100% to 5% AG significantly accelerated the first hour after crossing by +18.9 $\mu\text{m/hr}$ and +24.5 $\mu\text{m/hr}$ respectively, while neurons crossing from the high to intermediate density of 100% to 11% AG did not significantly accelerate. Additionally, although neurons accelerated when crossing from high to low AG densities this effect did not persist in all cases. Specifically, neurons significantly slowed by a net 25.2 $\mu\text{m/hr}$ the second hour after crossing from 100 to a LN only lane, while neurons crossing into 5% or 11% AG lane from 100% AG fields continued to sustain post crossing velocities into the second hour.

4. Discussion

4.1. DRGs upregulate functional integrins in a CSPG dose dependent manner

DRGs have been shown to upregulate integrins in response to outgrowth pathway CSPGs [21], but to our knowledge, integrin expression dynamics had not to this point been monitored directly in growth cones in a time-resolved fashion. Here we observed that DRGs upregulated $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin expression in growth cones in an AG dose dependent manner. It was deduced from these results that transient filopodia or lamellapodia sampling of AG boundaries in the case of LN only lanes was sufficient to induce integrin upregulation. A $\beta 1$ integrin block also demonstrated that all neurons seeded on AG patterns regardless of AG density upregulated functional integrins as evidenced by greater numbers of neurons recovering during and after blocking conditions, pronounced persistence in outgrowth, and improved adhesion during solution exchange versus neurons on non-patterned LN controls.

4.2. CSPG signaling pathway may slow DRG outgrowth independent of integrin expression

DRGs exhibited three distinct behaviors on the CSPG patterns generated for this study: 1) Outgrowth velocities on 7% thru 100% AG densities were robust at early timeframes but exhibited significant slowing by 12 hr post seeding, 2) Outgrowth on 2% and 4% AG lanes was robust at all time frames, resembling pathfinding dynamics observed in controls, and 3) Outgrowth on LN only lanes exhibited robust persistent outgrowth in the first timeframe then significant decreases in both persistence and velocity by the 9 – 12 hr and 12 – 15 hr time frames. In all cases, slowing in velocity and loss in persistence occurred despite continued robust integrin expression versus controls. Importantly, this slowing behavior triggered by transient CSPG sampling in LN only lanes was overcome by constant exposure to low level AG signals in 2% and 4% AG density lanes, indicating that removal of AG signals in outgrowth pathways after even brief AG contact causes this behavioral switch to occur in postnatal rat DRGs. These observations indicate that a secondary signaling pathway triggered by CSPGs either diminishes integrin participation in pathfinding over time or causes growth cones to slow despite continued integrin-LN anchoring.

By investigating the relationship of growth cone integrin expression density versus outgrowth velocities (Fig 3E), we found that although integrin density increased with increasing AG densities, the ratio of velocity per integrin density declined with increasing AG density, further demonstrating an effect of AG on integrin function. Furthermore, although AG clusters tend to reduce the adhesivity of outgrowth lanes as evidenced in our previous work [30], the low level of AG signal in most lanes was not expected to significantly impact LN availability, and thus loss of lane adhesivity can most likely be ruled out as a cause of diminished velocity to integrin density ratio. In related studies, integrin

dependent migration velocities and substratum attachment strength in non-neuronal cell types were demonstrated to be dependent on the number and density of integrin ligand contacts [31,32]. Conversely, our results confirm that neurons, unlike other migrating cell types, are able to not only modify integrin expression density in response to changes in the ECM environment to maintain outgrowth velocities but also are able to alter receptor activity. Finally, these results reveal exquisite neuronal growth cones possess over the control Local integrin expression in response to even minute changes in CSPG signals.

In light of our observations it is plausible that time of exposure and magnitude of AG signal in these experiments dictated the observed growth cone slowing response; AG signals in outgrowth lanes that at first caused significantly faster outgrowth velocities over a range of AG densities compared to controls switched to inhibition of robust outgrowth over time, indicating a time-dependent AG specific receptor mediated process (Fig 2A). Importantly, PTP σ was recently found to be a CSPG specific receptor expressed in DRGs that mediates neuronal response to proteoglycans [27]. When PTP σ -CSPG interactions were abolished, the inhibitory effects of AG on DRGs were greatly diminished although not completely removed, indicating the presence of one or more additional CSPG receptors in DRGs. In recent work, we demonstrated AG dose dependent upregulation in DRGs of CD44, a cell surface molecule important to neuronal pathfinding and differentiation in embryogenesis [33], which interacts with AG [34] and mediates adhesion to AG in leukocytes [35], providing further evidence of multiple CSPG receptors in DRGs.

4.3. CSPG signaling in nervous system development

Postnatal DRGs possess considerable pathfinding plasticity, and the observed slowing behavior of neurons on LN only lanes may lie in the normal response of DRGs to CSPG signals during development. There is ample evidence that DRG pathfinding is directed by CSPG barriers in the spinal cord during development [36,37,38]. Commissural and dorsal column axons are repelled by keratin and chondroitin sulfate proteoglycan expression in the floor plate but cross the floor plate where KSPGs and CSPGs are not expressed. Silver and Snow found that dorsal column axons grow in close apposition to the roof plate while commissural neurons do not [39]. Jhaveri and Hoffman-Kim further demonstrated that proteoglycan expression at the roof plate is down-regulated once commissural and dorsal column neurons reach their targets [40]. Additionally, retinal ganglion cells (RGCs) are directed by a transient CSPG barrier in the eye during development. Brittis et al. demonstrated that the CSPG barrier diminishes during development and coincides with RGC differentiation, and that RGCs differentiate prematurely if the CSPG barrier is artificially removed [36]. In another example, neural progenitor cell differentiation in rats is also prematurely initiated by early removal of CSPGs and is interestingly also dependent on integrin activation [41]. These in vivo observations of the developing CNS help explain the role of proteoglycans in CNS patterning, and perhaps provide evidence that the patterns created in this study have recapitulated some of the signals that are necessary for arresting DRG outgrowth during target innervation.

Curiously, growth cones on LN only lanes were the only test population that decreased dramatically in outgrowth velocity and persistent outgrowth trajectory, both of which are indicative of growth cones at a decision point [42]. This effect was further confirmed in AG step density assays in which neurons growing from high AG densities onto lanes devoid of AG slowed significantly compared to neurons continuing to encounter AG signals. These combined observations provide further evidence of a switching behavior triggered by a transient CSPG encounter that is reminiscent of behavior observed in other neurons during developmental pathfinding.

4.4. Implications of $\beta 1$ block assay on integrin expression dynamics

Blocking functional $\beta 1$ integrins in DRGs proved that growth cone migration was integrin dependent and that AG induced upregulation led to an enhanced ability to withstand and recover from $\beta 1$ integrin blocking conditions. We propose that neurons already expressing elevated levels of functional integrins were able to continue to extend growth cones during the block due to elevated membrane recycling activity. This was evidenced by neurons continuing to exhibit forward directed growth on LN only lanes and on 2% thru 7% AG lanes during the block unlike controls (Fig 4B). Neurons on AG patterns also exhibited greater substratum adhesion and recovery upon block removal (Fig 4A).

Growth cone behavior after block removal may also be an indicator of the ratio of active membrane bound integrins: intracellular sequestered integrins. For example, increased adhesion during block conditions and loss of forward growth after block for 31% and 100% AG neurons indicated that a greater proportion of the total active integrin pool was present in these growth cone membranes during block than in other neuron groups. Additionally, our observations indicated that neurons growing on other AG containing lanes retained a sufficiently large proportion of integrins in intracellular vesicles as to limit active integrin depletion and promote continued outgrowth post block. This behavior was perhaps best exemplified by 2% AG neurons, which exhibited robust outgrowth distances and significantly greater outgrowth persistence than controls and other AG pattern neuron groups by 3 hr post block.

4.5. Implications of AG step density assay on integrin expression dynamics

Evidence that substratum adhesivity or integrin expression levels in neurons and other cell types dictate migration speeds [18,31,32] should enable us to infer how quickly DRGs adapt integrin function after changes in substratum adhesivity or AG density. We observed that growth cones rapidly altered outgrowth speeds when crossing from high to low AG densities, reaching unusually high average velocities as fast as 69 $\mu\text{m/hr}$, as seen in the case of neurons growing from 100 to 5% AG (Fig 5B). These significant changes in velocity occurred over a timeframe of only 5 to 10 min after the crossing event, indicating a rapid ability to sense and adapt to outgrowth ECM changes. Findings in the literature point to a mechanism by which DRGs change velocity, namely Buettner and Pittman demonstrated that large changes in LN substratum concentrations are insufficient to change DRG outgrowth velocities or distances [43], while Lemons and Condit showed that changes in integrin activation state or intracellular cAMP levels can significantly alter DRG outgrowth speeds [22]. Thus, observed increases in DRG velocities on step density patterns were most likely not due to changes in substrate adhesivity but to a real change in the number or activation state of integrins expressed in growth cone membranes as triggered by an abrupt change in AG density.

5. Conclusion

We have demonstrated that postnatal rat DRGs supplied with NGF and LN rich substrata are able to initially regenerate past a wide range of CSPG barrier densities in a $\beta 1$ integrin dependent manner. However, at 12 hr post seeding, DRGs began to slow in outgrowth velocity largely as a function of increasing CSPG density, confirming the inhibitory nature of CSPGs. Additionally, growth cone integrin upregulation in response to AG patterns generated less contribution to outgrowth velocities per integrin density than observed in controls, suggesting a change in integrin function triggered by CSPG signals. It was found that transient growth cone sampling of CSPG boundaries was sufficient to cause functional integrin upregulation, but did not promote persistent growth on lanes devoid of CSPG clusters, while constant low levels of CSPG did, indicating a time delayed CSPG receptor

mediated response. In AG step boundary patterns it was confirmed that DRGs growing from high AG densities to LN only lanes accelerated then slowed in velocity over a 2 hr period while DRGs crossing from high to low AG densities accelerated then maintained velocities over the same 2 hr timeframe. These results indicate that CSPGs regulate both integrin expression and function in DRGs, integrin upregulation is insufficient to overcome CSPG densities greater than 8.9 ng/cm², and both CSPG density and spatiotemporal presentation have a profound effect on DRG pathfinding dynamics.

Acknowledgments

This work was supported by NIH grants to Hlady and Tresco. We thank Elena Budko for DRG isolation, Brian Baker for microfabrication assistance, Roy Biran and Clayton Underwood for cell culture and staining advice, Chris Rodesch for advice and assistance in cell imaging techniques, and Maureen Condic for suggestions on manuscript.

References

- [1]. Wallquist W, Zelano J, Plantman S, Kaufman SJ, Cullheim S, Hammarberg H. Dorsal root ganglion neurons up-regulate the expression of laminin-associated integrins after peripheral but not central axotomy. *J Comp Neurol*. 2004; 480:162–169. [PubMed: 15514929]
- [2]. Condic ML. Adult neuronal regeneration induced by transgenic integrin expression. *J Neurosci*. 2001; 21:4782–4788. [PubMed: 11425905]
- [3]. Condic ML, Snow DM, Letourneau PC. Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. *J Neurosci*. 1999; 19:10036–10043. [PubMed: 10559411]
- [4]. Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J. Regeneration of adult axons in white matter tracts of the central nervous system. *Nature*. 1997; 390:680–683. [PubMed: 9414159]
- [5]. Davies SJ, Goucher DR, Doller C, Silver J. Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *J Neurosci*. 1999; 19:5810–5822. [PubMed: 10407022]
- [6]. Houle JD, Tom VJ, Mayes D, Wagoner G, Phillips N, Silver J. Combining an autologous peripheral nervous system “bridge” and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *J Neurosci*. 2006; 26:7405–7415. [PubMed: 16837588]
- [7]. Tom VJ, Sandrow-Feinberg HR, Miller K, Santi L, Connors T, Lemay MA, et al. Combining peripheral nerve grafts and chondroitinase promotes functional axonal regeneration in the chronically injured spinal cord. *J Neurosci*. 2009; 29:14881–14890. [PubMed: 19940184]
- [8]. Lu P, Tuszynski MH. Growth factors and combinatorial therapies for CNS regeneration. *Exp Neurol*. 2008; 209:313–320. [PubMed: 17927983]
- [9]. Chau CH, Shum DK, Li H, Pei J, Lui YY, Wirthlin L, et al. Chondroitinase ABC enhances axonal regrowth through Schwann cell-seeded guidance channels after spinal cord injury. *FASEB J*. 2004; 18:194–196. [PubMed: 14630702]
- [10]. Liesi P, Kaakkola S, Dahl D, Vaheri A. Laminin is induced in astrocytes of adult brain by injury. *EMBO J*. 1984; 3:683–686. [PubMed: 6370690]
- [11]. Plantman S, Novikova L, Novikov L, Hammarberg H, Wallquist W, Kellerth JO, et al. Integrin messenger RNAs in the red nucleus after axotomy and neurotrophic administration. *Neuroreport*. 2005; 16:709–713. [PubMed: 15858411]
- [12]. Hammarberg H, Wallquist W, Piehl F, Risling M, Cullheim S. Regulation of laminin-associated integrin subunit mRNAs in rat spinal motoneurons during postnatal development and after axonal injury. *J Comp Neurol*. 2000; 428:294–304. [PubMed: 11064368]
- [13]. David S, Braun PE, Jackson DL, Kottis V, McKerracher L. Laminin overrides the inhibitory effects of peripheral nervous system and central nervous system myelin-derived inhibitors of neurite growth. *J Neurosci Res*. 1995; 42:594–602. [PubMed: 8568945]

- [14]. Jones LL, Sajed D, Tuszynski MH. Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. *J Neurosci.* 2003; 23:9276–9288. [PubMed: 14561854]
- [15]. Tucker BA, Rahimtula M, Mearow KM. Integrin activation and neurotrophin signaling cooperate to enhance neurite outgrowth in sensory neurons. *J Comp Neurol.* 2005; 486:267–280. [PubMed: 15844170]
- [16]. Zhou FQ, Walzer M, Wu YH, Zhou J, Dedhar S, Snider WD. Neurotrophins support regenerative axon assembly over CSPGs by an ECM-integrin-independent mechanism. *J Cell Sci.* 2006; 119:2787–2796. [PubMed: 16772333]
- [17]. Gardiner NJ, Fernyhough P, Tomlinson DR, Mayer U, von der Mark H, Streuli CH. Alpha7 integrin mediates neurite outgrowth of distinct populations of adult sensory neurons. *Mol Cell Neurosci.* 2005; 28:229–240. [PubMed: 15691705]
- [18]. Condic ML, Letourneau PC. Ligand-induced changes in integrin expression regulate neuronal adhesion and neurite outgrowth. *Nature.* 1997; 389:852–856. [PubMed: 9349817]
- [19]. Schmidt CE, Dai J, Lauffenburger DA, Sheetz MP, Horwitz AF. Integrin-cytoskeletal interactions in neuronal growth cones. *J Neurosci.* 1995; 15:3400–3407. [PubMed: 7751919]
- [20]. Lemons ML, Condic ML. Combined integrin activation and intracellular cAMP cause Rho GTPase dependent growth cone collapse on laminin-1. *Exp Neurol.* 2006; 202:324–335. [PubMed: 16899244]
- [21]. Domeniconi M, Filbin MT. Overcoming inhibitors in myelin to promote axonal regeneration. *J Neurol Sci.* 2005; 233:43–47. [PubMed: 15949495]
- [22]. Tom VJ, Steinmetz MP, Miller JH, Doller CM, Silver J. Studies on the development and behavior of the dystrophic growth cone, the hallmark of regeneration failure, in an in vitro model of the glial scar and after spinal cord injury. *J Neurosci.* 2004; 24:6531–6539. [PubMed: 15269264]
- [23]. Snow DM, Smith JD, Gurwell JA. Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. *J Neurobiol.* 2002; 51:285–301. [PubMed: 12150504]
- [24]. Snow DM, Letourneau PC. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J Neurobiol.* 1992; 23:322–336. [PubMed: 1624935]
- [25]. Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, Liu K, et al. PTP σ is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science.* 2009; 326:592–596. [PubMed: 19833921]
- [26]. Hodgkinson G, Hlady V. Relating material surface heterogeneity to protein adsorption: I. The effect of annealing of micro-contact-printed OTS patterns. *J Adhes Sci Technol.* 2005; 19:235–255. [PubMed: 19693285]
- [27]. Hayashi I, Sato GH. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature.* 1976; 259:132–134. [PubMed: 813153]
- [28]. Jamasbi RJ, Kennel SJ, Stoner GD. A monoclonal antibody produced against a rat esophageal carcinoma cell line reacts with an integrin-like molecule expressed by rat epithelial cells. *Hybridoma.* 1992; 11:581–594. [PubMed: 1459582]
- [29]. Hodgkinson GN, Tresco PA, Hlady V. The differential influence of colocalized and segregated dual protein signals on neurite outgrowth on surfaces. *Biomaterials.* 2007; 28:2590–2602. [PubMed: 17316787]
- [30]. Hodgkinson GN, Tresco PA, Hlady V. The influence of sub-micron inhibitory clusters on growth cone substratum attachments and CD44 expression. *Biomaterials.* 2008; 29:4227–4235. [PubMed: 18694596]
- [31]. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature.* 1997; 385:537–540. [PubMed: 9020360]
- [32]. DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J Cell Biol.* 1993; 122:729–737. [PubMed: 8335696]

- [33]. Lin L, Chan SO. Perturbation of CD44 function affects chiasmatic routing of retinal axons in brain slice preparations of the mouse retinofugal pathway. *Eur J Neurosci.* 2003; 17:2299–2312. [PubMed: 12814363]
- [34]. Fujimoto T, Kawashima H, Tanaka T, Hirose M, Toyama-Sorimachi N, Matsuzawa Y, et al. CD44 binds a chondroitin sulfate proteoglycan, aggrecan. *Int Immunol.* 2001; 13:359–366. [PubMed: 11222505]
- [35]. Murai T, Sougawa N, Kawashima H, Yamaguchi K, Miyasaka M. CD44-chondroitin sulfate interactions mediate leukocyte rolling under physiological flow conditions. *Immunol Lett.* 2004; 93:163–170. [PubMed: 15158613]
- [36]. Brittis PA, Canning DR, Silver J. Chondroitin sulfate as a regulator of neuronal patterning in the retina. *Science.* 1992; 255:733–736. [PubMed: 1738848]
- [37]. Höke A, Silver J. Proteoglycans and other repulsive molecules in glial boundaries during development and regeneration of the nervous system. *Prog Brain Res.* 1996; 108:149–163. [PubMed: 8979800]
- [38]. Oakley RA, Tosney KW. Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. *Dev Biol.* 1991; 147:187–206. [PubMed: 1908800]
- [39]. Snow DM, Steindler DA, Silver J. Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. *Dev Biol.* 1990; 138:359–376. [PubMed: 1690673]
- [40]. Jhaveri S, Hoffman-Kim D. Unilateral containment of retinal axons by tectal glia: a possible role for sulfated proteoglycans. *Prog Brain Res.* 1996; 108:135–148. [PubMed: 8979799]
- [41]. Gu WL, Fu SL, Wang YX, Li Y, Lü HZ, Xu XM, et al. Chondroitin sulfate proteoglycans regulate the growth, differentiation and migration of multipotent neural precursor cells through the integrin signaling pathway. *BMC Neurosci.* 2009; 10:128. [PubMed: 19845964]
- [42]. Mason CA, Wang LC. Growth cone form is behavior-specific and, consequently, positionspecific along the retinal axon pathway. *J Neurosci.* 1997; 17:1086–1100. [PubMed: 8994063]
- [43]. Buettner HM, Pittman RN. Quantitative effects of laminin concentration on neurite outgrowth in vitro. *Dev Biol.* 1991; 145:266–76. [PubMed: 2040373]

Figure 1.

CSPG patterns and growth cones. A - Schematic image of repeating AG pattern (grey) on LN background (white). Repeating LN only lanes and AG cluster patterned lanes were 30 μm wide, separated by 100 μm wide 100% AG fields. B – Fluorescence images of anti-α6β1 integrin stained DRGs (green) on AG patterned (red) on LN coated substrata (black). Outgrowth lanes consisted of LN only and 2% to 31% average AG lane densities. Growth cones grew over AG clusters, but evidence of integrin localization over LN sites can be seen in growth cones on 4% and 7% AG lanes. Growth cones were unusually large at initial time frames, especially on LN Lanes and at low AG densities, but diminished in size on average over time. Scale bars are 20 μm for all images.

Figure 2.

DRG growth cone pathfinding dynamics on AG patterns and LN only controls. A – Mean growth cone velocities over four time periods expressed as the difference from initial timeframe control neurons. Neurons slowed over time at higher AG densities, but also on LN Lanes. * Significant difference from controls, $p < 0.001$ KW group analysis, $p < 0.032$ MW pair-wise comparisons; ** Significant difference from initial timeframe for given substratum condition, $p < 0.028$ KW group analysis, $p < 0.016$ MW pair-wise comparisons; + Significant difference from LN Lane neurons, $p < 0.001$ KW group analysis, $p < 0.020$ MW pair-wise comparisons. B – Number of growth cone movements (n) on each substratum monitored for outgrowth velocity analysis. C – Mean outgrowth persistence over four time periods expressed as the difference from initial timeframe control neurons. Neurons on low density AG lanes exhibited constrained persistent outgrowth compared to controls for most timeframes. However, LN Lane neurons uniquely exhibited significant loss in persistence over time compared to controls. * Significant difference from controls, $p < 0.005$ KW group analysis, $p < 0.05$ MW pair-wise comparisons; ** Significant difference from initial timeframe for given substratum condition, $p < 0.000$ KW group analysis, $p < 0.009$ MW pair-wise comparisons; + Significant difference from LN Lane neurons, $p < 0.026$ KW group analysis, $p < 0.032$ MW pair-wise comparisons. D – Number of growth cone movements (n) for each substratum monitored for outgrowth persistence analysis. All error bars are $\pm 95\%$ confidence intervals of absolute mean velocity values. N – insufficient data.

Figure 3.

DRG growth cone integrin expression density and function. A – $\alpha1\beta1$ integrin growth cone expression density normalized to initial timeframe control neurons. Only LN Lane, 7% AG, and 31% AG neurons significantly upregulated $\alpha1\beta1$ integrin expression versus controls, and only at the second time point. * Significantly greater than control neurons, $p < 0.040$ ANOVA, $p < 0.036$ t-test pair-wise comparisons; + Significantly greater than LN Lane neurons, $p < 0.029$ ANOVA, $p < 0.036$ t-test pair-wise comparisons. B – Number of growth cones (n) on each substratum imaged for $\alpha1\beta1$ integrin expression. C – $\alpha6\beta1$ integrin growth cone expression density normalized to initial timeframe control neurons. Integrin expression increased in general as a function of increasing AG substratum density. * Significantly greater than control neurons, $p < 0.044$ ANOVA, $p < 0.032$ t-test pair-wise comparisons; – Significantly less than 100% AG neurons, $p < 0.044$ ANOVA, $p < 0.035$ t-test pair-wise comparisons. D – Number of growth cones (n) imaged on each substratum for $\alpha6\beta1$ integrin expression. E – Growth cone velocity over time as a function of $\alpha6\beta1$ growth cone expression density and substratum condition. The ratio of velocity to integrin density diminished in general with increasing AG density, indicating perhaps a lower contribution of integrin receptors to locomotion at higher AG densities. LN Lane neurons also exhibited lower velocity to integrin density ratios than controls despite of the absence of AG clusters in these lanes. $\pm 95\%$ confidence intervals for mean ratios were calculated using the EC Fieller method. N – insufficient data.

Figure 4.

DRG outgrowth response to functional $\beta 1$ integrin block. A – DRG recovery from 1 hr antibody block conditions. DRG cell bodies and growth cones exhibited robust attachment to substrata containing AG signals compared to LN only controls. B – Average and direction of straight line outgrowth during and after block conditions. Large error bars during block conditions indicate the varied responses of growth cones, which included stalling and complete collapse. On average, most control growth cones collapsed or retracted during the $\beta 1$ integrin receptor block while growth cones on AG patterns did not. Most neuron groups exhibited robust recovery in outgrowth after removal of the blocking antibody, with controls, LN Lane, and 7% AG neurons exhibiting significant improvement in outgrowth. + Significantly different from outgrowth during block, $p < 0.046$ KW group analysis, $p < 0.035$ MW pair-wise comparisons. C – Outgrowth persistence during and after block conditions. Many neuron groups including LN Lane neurons exhibited significantly greater outgrowth persistence than controls either during or shortly after block conditions. By the final time frame, 2% AG neurons exhibited significantly greater outgrowth persistence than other neuron groups. * Significant difference from controls and three control neurons, $p < 0.05$ KW group analysis, $p < 0.05$ MW pair-wise comparisons; + Significantly different from outgrowth during block, $p < 0.017$ KW group analysis, $p < 0.035$ MW pair-wise comparisons. § Significantly less than 2% AG neurons, $p < 0.016$ KW group analysis, $p < 0.03$ MW pairwise comparisons. D – Number of growth cones (n) imaged on each substratum for outgrowth distance and persistence analyses. All error bars are $\pm 95\%$ confidence intervals of means. N – insufficient data.

Figure 5.

Aggrecan step density patterns and DRG outgrowth behavior. A – Fluorescence images of AG step density patterns (green) on LN background (black). Scale bar, 20 μm . B – Change in DRG growth cone outgrowth velocity after crossing various AG step density boundaries. Neurons in general accelerated after crossing from high to low AG density fields. However, by one hour post crossing, LN Lane neurons significantly decelerated, while neurons crossing into 5% AG lanes did not. * Greater than pre boundary crossing velocity, $p < 0.018$ KW group analysis, $p < 0.032$ MW pair-wise comparisons; ** Significantly less than first hour post boundary crossing velocity, $p < 0.01$ KW group analysis, $p < 0.01$ MW pair-wise comparisons. Absolute mean growth cone velocities are listed in parentheses for select conditions. D – Number of growth cone movements (n) for each substratum monitored for outgrowth velocity analysis. All error bars are $\pm 95\%$ confidence intervals of absolute mean velocity values.

Table 1

Aggrecan Pattern Characterization

Substrate	Average AG Density (ng/cm ²)	Actual % AG Density*	Average Single Cluster area (μm ²)	Average AG Molecule/Cluster
LN Lane	0	-	-	-
2%	3.74 ± 0.59	1.51	0.51	1.4
4%	8.86 ± 1.24	3.57	0.52	1.6
7%	16.2 ± 1.82	6.53	0.51	1.4
11%	27.0 ± 2.93	10.9	0.53	1.5
31%	77.2 ± 7.40	31.1	0.89	3.4
100%	248 ± 11.0	100	-	-

* Aggrecan lane density expressed as percentage of AG field density between outgrowth lanes. All AG densities were significantly different from all others. p<0.001 ANOVA, p<0.001 by t-test for all comparisons. ±95% confidence intervals.

Table 2

Step Boundary Aggrecan Pattern Characterization

Substrate	Average AG Density (ng/cm ²)	Actual % AG Density*	Average Single Cluster area (μm ²)	Average AG Molecule/Cluster
LN Lane	0	-	-	-
3%	6.84 ± 0.74	2.76	0.54	1.5
5%	11.4 ± 1.65	4.60	0.45	1.3
11%	28.1 ± 4.16	11.4	0.54	1.5

* Aggrecan lane density expressed as percentage of AG field density between outgrowth lanes. All AG densities were significantly different from all others. p<0.001 ANOVA, p<0.001 by t-test for all comparisons. ±95% confidence intervals.