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The influence of sub-micron inhibitory clusters on growth cone substratum attachments and CD44 expression

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Abstract

Proteoglycan expression patterns in the central nervous system guide neuronal pathfinding during development, but also disrupt regeneration after injuries. To deepen our understanding of the molecular level effects of proteoglycan spatial arrangements on neuronal pathfinding, we designed micropatterning stamps for the precise placement of near single molecule chondroitin sulfate proteoglycan (CSPG) clusters into regularly spaced arrays. Actin ultrastructural analysis in dorsal root ganglion neurons grown on laminin-coated substrata patterned with aggrecan cluster arrays revealed filopodial and lamellapodial edge contact avoidance of individual clusters, while growth cone lamellapodia and central domains were able to span multiple clusters over a range of cluster densities. Total internal reflection fluorescence microscopy interrogation of growth cone substratum morphology further revealed persistence of integrin mediated substratum adhesion and local out-of-plane membrane bending over clusters on the height scale of aggrecan glycosaminoglycan side chains. Direct imaging of cell adhesion molecule CD44 expression in growth cones revealed an aggrecan dose dependent upregulation in CD44 molecules. Evidence of CD44 clustering coinciding with underlying aggrecan molecules imply CSPG-CD44 interactions. The results reveal the limited local repulsive effect of CSPGs on neuronal structures and provide evidence that CD44 upregulation in neurons is affected by local CSPG expression.

1. Introduction

Central nervous system (CNS) patterning is directed by a series of spatial-temporal events, which depend on the interplay of soluble and bound protein expression and cellular responses. In particular, neurons in a process of pathfinding, integrate multiple extra cellular matrix (ECM) cues through the motile growth cone (GC) structure [1,2]. Integrated signals are processed and transduced by the GC, resulting in pathfinding changes and GC morphologies, which have been linked to directed outgrowth or pathway decision behavior [3,4].

During development, a number of sensory neurons encounter both permissive and inhibitory cues composed of laminin tracts (LN) bounded by molecular barriers in the form of chondroitin sulfate proteoglycans (CSPGs). For instance, commissural and dorsal column axons are repelled by keratin (KSPG) and chondroitin sulfate proteoglycan expression in the roof plate, but cross the floor plate where KSPGs and CSPGs are not expressed [5,6]. It is also known

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Competing Interests Statement

The authors declare that they have no competing financial interests.

that retinal ganglion cell (RGC) development depends on CSPG expression in the retina as demonstrated by premature RGC differentiation after artificial CSPG removal [7].

Numerous in vitro assays have been devised to elucidate the interaction between bound permissive and inhibitory signals and their effect on neuronal outgrowth response and membrane receptor expression. It has been demonstrated that neuronal adhesion and migration on permissive substrates require sufficient integrin receptor expression levels, while the nature of migration depends on receptor number and activity state [8–10]. Although there is evidence of the existence of CSPG receptors, the mechanism by which neurons interact with CSPGs remains unclear. For example, neurons exhibit intracellular calcium transients upon CSPG contact, [11] and the inhibitory effects of CSPGs on neuronal outgrowth can be diminished by blocking certain second messenger systems [12]. We are particularly interested in the cell adhesion molecule CD44, which exhibits properties indicative of a proteoglycan receptor. CD44 is upregulated in various cell types following CNS injury including neurons [13]. Additionally, CD44 expression plays a role in developmental pathfinding, and in several studies has been shown to bind to CSPGs including versican and aggrecan (AG) [14–16].

To date, nearly all in vitro pathfinding assays involving patterning have utilized either diffuse colocalized inhibitory and permissive protein fields or sharp inhibitory protein field boundaries on permissive layers. These assays have provided evidence that integrin receptor expression is upregulated in response to CSPGs, ultrastructural actin and microtubule organization change during growth cone turning, and outgrowth dynamics are altered at various CSPG boundaries [17–21]. However, the lack of spatial precision with which CSPGs could be placed or patterned in previous studies limited the spatial resolution at which neuronal interactions with CSPGs could be observed. Thus, the available data in the field of inhibitory molecules raises several important questions including: (1) what is the molecular basis of CSPG inhibition that affects neuronal outgrowth and attachment, (2) at what length scales do spatial changes in CSPG expression density alter pathfinding behavior, and (3) what is the minimum CSPG surface density that results in growth cone morphology and behavior changes?

A number of researchers have turned to ‘soft lithography’ surface patterning techniques to investigate cell attachment and morphology behavior in the presence of precisely placed multiple environmental cues [22,23]. In past studies, such techniques were used to create substrates containing regularly spaced integrins presented as islands. Cell attachment to the integrin patterns provided evidence that phenotypic expression and apoptosis depend on integrin spatial arrangement and availability. It was also demonstrated that integrin island placement dictated mechanical cellular responses [24].

To study the molecular and spatial expression effects of CSPGs on neuronal pathfinding, we devised soft lithography micropatterns to produce various densities of regularly spaced sub-micron aggrecan clusters on a LN-coated substrate. Differing, well-defined AG cluster densities were separated into parallel outgrowth lanes by uniform AG fields so that neuronal outgrowth could be directed across various mixed fields of AG and LN on a single substrate. Dorsal root ganglion (DRG) neurons were visualized at several stages of outgrowth and interrogated for changes in morphology, cell-substratum adhesion behavior, and CD44 expression as a function of AG density.

By precisely controlling the spatial arrangements of AG clusters, we were able to interrogate neurite outgrowth behavior in response to protein signal heterogeneity both on the size scale of the aggrecan molecule and on a size scale comparable to GC structures. Thus, GC and neurite interactions with AG at the molecular level were analyzed to more fully understand the nature of CSPG inhibition and neuronal response. Total internal reflection fluorescence microscopy (TIRFM) was additionally employed to visualize the nature of neuron substratum interactions

over individual AG clusters. From our observations, we demonstrate that neurite branching and outgrowth orientation were affected by large-scale pattern features, while GC structures exhibited sensitivity to individual AG clusters. In addition, we demonstrate that DRG CD44 expression is linked to substratum AG density.

2. Materials and Methods

2.1. Micropattern device fabrication

Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) micropatterning devices were designed, modeled, and fabricated utilizing the University of Utah Microfabrication Laboratory. Patterns modeled in Autocad R14 (Autodesk) software were translated onto photolithographic masks (Nanofilm) by use of a pattern generator and image repeater (Electromask). Resulting photolithographic masks were used directly as molds to produce PDMS stamps. Prepolymer PDMS was poured into a mold and cured at 100°C for 90 minutes. Stamps were designed to generate a 1×1 cm² pattern of six repeating, parallel 30 µm-wide LN coated lanes separated by 100 µm-wide AG coated fields.

2.2. Protein preparation and dye conjugation

Englebreth-Holm-Swarm mouse tumor laminin (Invitrogen) and bovine articular cartilage aggrecan (Sigma) were labeled with Alexa Fluor (AF) AF488 using protocols provided by Invitrogen. LN in Tris buffer as received from Invitrogen was exchanged to PBS buffer using a PD-10 desalting column before labeling. Labeling reactions of a mixture of 500 µl of 2 mg/ml AG in PBS (or 2.75 ml of 375 µg/ml LN in PBS) + 0.25 mg of reactive dye + 10:1 (v:v) protein solution to 1 M Sodium Bicarbonate buffer at pH 8.3 were carried out for 1 hour at room temperature with mild stirring. Alternatively, AF594 was mixed with AG 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 (GE Healthcare) packed glass columns. Final protein solution concentrations and dye to protein molar ratios were determined through absorbance spectroscopy using the Beer-Lambert law and based on the extinction coefficients for the specific dyes and proteins used in this study.

2.3. Surface patterning

Glass coverslips were cleaned prior to surface modification in a 1 H₂O₂ : 2 H₂SO₄ mixture, rinsed 4x with double distilled deionized (DD) water, and dried under a nitrogen stream. 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 AG (1 mg/ml) was applied to PDMS devices for 20 minutes. After adsorption, glass coverslips and PDMS stamps were rinsed with DD water and briefly dried under a nitrogen stream. AG patterns were transferred onto adsorbed LN through microcontact printing (µCP) in which AG-coated PDMS stamps were brought into conformal contact with LN-coated coverslips. “LN control” surfaces containing adsorbed LN without stamped AG patterns served as negative controls.

2.4. Protein pattern density determination

A fluorescence microscopy imaging technique described in previous work [25] was utilized to determine absolute AG and LN protein surface densities. Laminin surface density was determined from three samples imaged at 8 to 10 locations each. Aggrecan surface densities were determined for AF488-AG patterns stamped on non-labeled LN-coated glass coverslips. Three AG patterned samples were produced and imaged at 8 to 10 locations for each distinct AG pattern density in each sample. The fluorescence imaging technique used to quantify protein densities was sufficiently sensitive to distinguish differences in AG densities at a lower resolution limit of 5.1 ng/cm².

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 day 1–2 Fisher rat DRG neurons were explanted then cleaned and purified. Ganglion bodies were taken from all accessible levels of the vertebral column then placed in Leigovitz's L-15 medium (Gibco) 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 of purified O4 antibodies to a 1ml cell suspension in DMEM then by the addition of 50 μ l of purified rabbit complement. Cell supernatants were resuspended in serum free SATO⁻ medium [26] containing 10% DMSO and placed in liquid nitrogen storage for later use.

All patterned substrates were treated with 500 μ g/ml gentamicin in sterile PBS for 15 to 30 minutes then rinsed 3x 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 for cell staining experiments and at 2k viable cells/sample for TIRFM experiments. Before cell staining, DRGs were fixed in 2% paraformaldehyde, rinsed 3x, and stored refrigerated in PBS containing 0.1% NaN₃.

2.6. Dynamic filopodia and growth cone imaging

Filopodia sampling of and growth cone navigation through mixed AG and LN patterns were imaged by time lapse video microscopy using a Nikon Diaphot 200 microscope equipped with a sample cell holder and Biopetechs sample heater, Princeton Instruments CCD camera, Nikon 100x Plan Apo, 1.4 N.A., phase 4 objective, and an automated microscope stage controlled by OpenLab v3.1.5 software. Images of growth cones were captured every 4 seconds for several hours of outgrowth.

2.7. Growth cone substratum contacts

DRG growth cone and cell body surface contacts on mixed AG-LN patterns were imaged through prism based TIRFM. The TIRFM set-up, which was designed and built in-house, consisted of a tunable Ar⁺ ion laser (American Laser Corp) launched into an optical fiber (Oz Optics) with a terminating GRIN lens, which was used to focus a 30 μ m diameter laser spot onto a sample surface through a 70° angle dovetail cut quartz prism. The sample and prism holder were custom designed and machined to fit the stage of a Nikon E400 Eclipse upright microscope.

Glass coverslip samples containing live DRGs loaded with 5 μ M Calcein AM (Invitrogen) and adherent to the AG-LN surface patterns were optically coupled to the prism by oil immersion then enclosed in a 50 μ m thin chamber containing fresh cell growth media using an elastomeric gasket (Specialty Silicone Products) and #1 coverslip (Fisher Scientific) as a chamber lid. Evanescent field excited fluorescence or epifluorescence emission was collected by use of a 100x, 1.35 N.A. objective oil-coupled to the chamber lid. A 5% evanescent field excitation intensity limit was estimated at 450 nm separation distance from the cell growth substratum according to equations presented by Axelrod and Burmeister et al. [27,28].

2.8. CD44 expression and actin organization

DRGs seeded on AG cluster patterns were fixed 6 to 15 hours post-seeding. After staining for CD44 or actin, samples were mounted in Fluoromount G (Southern Biotech) on glass slides

for imaging. For CD44 imaging, cells were incubated in 10 µg/ml mouse anti-rat CD44 (BD Pharmingen) in 4% goat serum + 0.3% Triton for 1 hour, rinsed 3x in PBS, incubated in 1:500 v:v goat anti-mouse AF488-IgG1 (H&L) (Invitrogen) in 4% goat serum + 0.3% Triton for 1 hour, then rinsed 3x in PBS. DRG growth cone CD44 expression was imaged and quantified on two samples at each of four different outgrowth time points for a total of eight samples.

Actin staining was accomplished by incubating DRGs in 0.1% Triton for 10 minutes, rinsing 3x with PBS, then incubating in AF488-phalloidin (1:40 v/v Invitrogen) for 90 minutes at 4° C. Expected random colocalization of filopodia and AG clusters was calculated from the product of red AG cluster and green filopodial actin filament pixel area coverage, while actual co-localization was calculated as a percentage of image area occupied by both red and green channels. Neurite branching angles were determined at distinguishable branch points for neurites greater than 10 µm in length. Neurite segment outgrowth orientation angles with respect to LN lane edges ($\pm 90^\circ$) were measured for straight segments greater than 10 µm in length. See Fig. 2D for schematic representation of neurite angle measurements. Filopodia–AG colocalization, neurite branching angles, and neurite segment outgrowth orientation were measured for cells on two samples at each of four different outgrowth time points for a total of eight samples.

2.9. Statistical analysis

Statistical analysis was performed with SPSS v12.0 software. Data sets were checked for normality by fitting cumulative proportion plots to Gaussian distributions. One-way ANOVA was conducted for each group of data to ensure statistical significance between multiple cases before conducting comparisons. T-tests for multiple comparisons were two-tailed and calculated assuming equal variance unless otherwise noted. Statistical outliers in CD44 density data were omitted from analysis.

3. Results

3.1. Protein patterns

Microcontact printing was used to generate precise AG cluster patterns on LN-coated coverslips. The resulting patterns consisted of a repeating set of six parallel 30 µm-wide lanes separated by 100 µm-wide uniform AG fields. The first lane in each set consisted of pure LN, while each of the five remaining lanes contained different densities of hexagonally spaced AG clusters on LN (Fig. 1). LN-coated substrates without AG patterns were additionally created as negative controls (LN control). LN was coated on all samples as a growth permissive background layer at an average density of 334 ± 53 ng/cm² ($\pm$ stdev).

Close to 100% protein transfer rates during AG stamping were achieved as evidenced by PDMS surface imaging and by few defects in AG patterns. Uniform AG fields were transferred onto LN-coated substrata at a density of 248 ± 11 ng/cm², or 6.1 AG molecules/µm². Clusters in the four least dense AG surface patterns were transferred at an average density of 1.5 AG molecules/cluster and at an average cluster area of 0.51 µm². Clusters in the most densely packed AG arrays were transferred at 3.5 AG molecules/cluster on average and at an average cluster area of 0.89 µm². The average AG density for each lane is listed in Table 1. Individual AG molecules at 400 nm in length were visible by fluorescence microscopy imaging both in homogeneous layers and in AG clusters (Fig. 1C–E). Figure 1, panels C–E demonstrate similarities in AG molecular morphology for stamped and adsorbed uniform AG layers as well as for stamped AG clusters.

It was observed that AG molecules were patterned at a lower density in individual clusters than in fields, presumably due to differential adsorption behavior on 1 µm tall stamp pillars vs.

planar PDMS surfaces (Fig. 1). Indeed, the length of a single AG molecule is approximately half that of the height or diameter of the PDMS stamp pillars used in this study. Consequently, some fraction of AG molecules would presumably adsorb to pillars in orientations that spanned both pillar face and wall, affecting packing and transfer densities. AG adsorption density on PDMS stamps was higher than on glass presumably because of strong hydrophobic interactions at stamp surfaces not present at glass interfaces. Thus, stamping could be used to transfer higher densities of AG onto glass than would normally occur through direct adsorption.

3.2. DRG growth cone morphology and neurite branching patterns

Actin cytoskeletal filaments are central to neuron GC morphology and motility. Hence, they serve as convenient markers of such neuronal structures as GC filopodia, lamellapodia, and neurites. Actin staining revealed that DRG cell body attachment and outgrowth were predominately constrained to LN lanes regardless of the presence or coverage of AG clusters within the adhesive terrain. Occasionally, however, pathfinding neurites crossed into uniform AG fields, providing an opportunity to study DRG outgrowth on the highest (100%) AG density.

Actin staining and time-lapse phase microscopy revealed varied behavior among different GC structures and neurites (Fig. 2&Fig. 3). By computing the expected random probability of actin and AG cluster colocalization for filopodia, it was found that filopodia preferentially adhered to LN sites vs. AG sites for all but the 11% AG density lanes (Fig. 2E, $p < 0.04$, t-test). Confirming observations of fixed neurons, time-lapse imaging of a pathfinding growth cone demonstrated filopodial sampling of AG and LN sites but preferential adhesion to LN sites prior to GC extension, which typically continued between AG clusters (Fig. 3). Actin-stained GC lamellapodial edges were also observed, in general, to stop at AG cluster boundaries, providing evidence of contact avoidance. Conversely, actin organization in GC core regions was not noticeably affected by the presence of AG clusters as evidenced by a majority of GCs spanning several, or in some cases, numerous AG clusters (Fig. 2A–C). Interestingly, neurites did not discriminate between AG clusters and LN adhesion sites (Fig. 2C), suggesting that their behavior was modified after growth cone extension. In support of this observation, neurite orientation was significantly constrained by LN lane boundaries ($p < 0.001$, t-test), but not by AG cluster alignment (Fig. 2F). If neurite orientation had been affected by AG clusters, we would have expected to observe alignment with one or more of the hexagonal directions presented by the AG arrays. Neurite branching angle was significantly reduced in neurons growing on AG bounded lanes compared to neurites on LN control surfaces with no AG patterns. ($p < 0.012$, t-test). As with neurite segment orientation, neurite branching was not affected by AG cluster density or organization (Fig. 2G) but was influenced by the LN lane AG boundaries.

3.3. Growth cone substratum contact morphology and CSPG avoidance

TIRF imaging is a surface sensitive technique, which enables the observation of membrane separations from the growth substratum on a tens of nm height scale in viable cells [28]. TIRF imaging of DRGs thus revealed the nature of cell body, neurite, and GC attachments over mixed LN and AG patterns at a more localized or site specific perspective (Fig. 4). It was found that GC and filopodia structures were generally in close apposition to the substratum, whereas neurites were less often completely attached to the substratum as demonstrated in Fig. 4A, further suggesting reduced sensing and adhesion function in neurites. In agreement with filopodia contact avoidance behavior, we found that membrane patches in GCs and cell bodies also appeared to avoid AG molecules by arching over the patterned clusters. Despite the presence of numerous AG clusters, integrin mediated substratum contacts persisted as evidenced by close contacts to LN sites between adjacent AG molecules spaced less than 1 μm apart.

3.4. CD44 Expression

In a search for links between the cell adhesion molecule CD44 and CSPG expression observed in CNS injuries, pathfinding DRGs were grown on AG cluster patterns for various post-seeding time intervals, then fixed and labeled for CD44 expression. Immunolabeled CD44 molecules in intact GCs were imaged to ascertain the local effect of AG density on expression. By calculating the relative levels of CD44 expression on a GC area basis, it was found that CD44 expression density increased over time in an AG dose dependent manner (Fig. 5D). Average expression levels increased by as much as 400% and were most pronounced in GCs growing on 31% and 100% AG density lanes after 15 hours post-seeding. CD44 expression increased significantly vs. LN only controls at 15 hours post-seeding for all neurons growing on AG patterns except for the 4% AG density lane ($p < 0.02$, t-test).

In general, there was no apparent spatial pattern of CD44 expression in DRG structures, although staining of both membrane-bound and intracellular CD44 molecules may have obscured our ability to detect such organization. However, we did find one example of CD44 expression in clusters, which were colocalized with the underlying AG molecule surface pattern (Fig. 5B&C). The hexagonally arranged CD44 clusters were most apparent in lamellapodia extending from a cell body just beginning to project neurites into a lane of AG clusters (Fig. 5B).

4. Discussion

A wealth of knowledge of the effects of proteoglycans on neuronal outgrowth has been garnered through in vitro pattern assay design and observation. However, due to difficulty in controlling the spatial placement of individual inhibitory molecules with precision, the nature of CSPG inhibition on the molecular scale has remained elusive. For example, we and others have demonstrated in stripe assays that bounding AG fields restrain neurite growth to LN lanes due to contact avoidance of AG fields [18–21,25]. However, at sufficiently high LN:CSPG ratios, neurites grow through mixed CSPG-LN fields and are able to adapt to increasing CSPG concentrations, demonstrating that CSPGs are weak inhibitors to neurite outgrowth [21,25, 29,30].

In previous work, we demonstrated that pure AG lanes significantly restrained DRG cell body attachment and neurite outgrowth to LN fields to a greater extent than both pure non-permissive albumin lanes and mixed AG-LN lanes, suggesting AG is intrinsically inhibitory [25]. It was also found that AG molecules placed on substrata by microcontact printing retained inhibitory properties comparable to that produced by other patterning methods, and that both the core protein and chondroitin sulfate chains contributed to the inhibitory effect [25].

4.1. DRG lateral sensing of aggrecan

By observing DRG pathfinding behavior on AG cluster patterns, we found that filopodia discriminate between LN and AG sites on the length scale of one to several AG molecules, demonstrating the spatial precision of proteoglycan sensing in GCs. These observations also corroborate other evidence that filopodia act as sensor organs during growth cone pathfinding. Conversely, on all AG surface densities, growth cone substratum attachment was seemingly less sensitive to the presence of AG molecules, and growth cones often spanned multiple proteoglycan clusters. Our ability to pattern near single molecule aggrecan allowed this unique observation, indicating perhaps a requirement of close substratum adhesion for GC motility when navigating areas of CSPG expression, but not required for filopodia movement. Furthermore, the diffuse nature of adsorbed or stamped AG layers on LN observed in this (Fig. 1C&D) and our previous study [25] suggest that GCs are able to attach to exposed LN sites for various AG densities ranging from 4 to 248 ng/cm². Additionally, we found that neurites

did not demonstrate adhesion discrimination between AG and LN sites, suggesting a lower relative contribution of sensing and adhesion functions in these structures.

4.2. DRG out-of-plane response to aggrecan clusters

Although GC spanning of AG clusters observed in actin staining seemed to indicate less sensitivity to proteoglycans than observed for filopodia and lamellapodia, TIRF imaging clearly demonstrated out-of-plane membrane bending and possible contact avoidance where GC membranes arched over individual AG clusters. The unique nature of the TIRF images led to the finding that DRGs are able to navigate dense layers of CSPGs because of an ability to continue to form integrin mediated substratum adhesion even in patches between proteoglycan molecules less than 1 μm apart. Furthermore, a number of TIRF images revealed that both growth cones and cell bodies formed numerous close contacts with the substratum so that membrane bending over AG clusters was observed for nearly the entire imaged cell structures (Fig. 4A,C,&D). The extent of membrane contacts observed in the TIRF images are unusual for laminin mediated adhesion in DRG growth cones, which typically form less well defined and fewer close substratum contacts [31]. This change in substratum adhesion behavior appears to be part of a compensatory mechanism that enables growth cones to navigate AG covered LN despite an apparent inability to form integrin contacts through AG clusters.

In addition to revealing how GC attachments adapt to CSPG covered substrata, these unique observations confirm the weak repulsive nature of proteoglycans on neuronal adhesion. When we take in account the molecular structure of aggrecan, we gain additional insights into the possible mechanisms of CSPG repulsion. By analyzing short-scale lateral variations in the evanescent field-excited fluorescence caused by local membrane bending, we were able to compare the relative membrane separation distances over adjacent LN adhesion sites and AG clusters. By assuming a membrane-substratum separation of 15 nm for fluorescent intensities associated with LN-integrin receptor contacts [32], we found that membranes were separated from the substratum by on average ~ 36 nm over AG clusters, considerably greater than over the LN exposed sites. It has been demonstrated that extended AG glycosaminoglycan (GAG) chains are 26 ± 7 nm in length as measured by scanning force microscopy (SFM) [33], and in previous SFM work, we found that stamped AG core proteins measured in air or in water had a height of 7 nm [25]. These combined measurements coincide with our TIRF image calculations and suggest that DRG membranes arch over AG molecules at the height of fully extended GAG chains in an aqueous environment. This evidence of close membrane apposition to CSPG molecules points to an anti-adhesive mechanism generated by volume exclusion and steric hindrance of GAG chains. Indeed, in mechanical studies of articular AG, it has been shown that glycosaminoglycan chains generate mechanical repulsion on the molecular level through volume exclusion [34]. Close apposition of the GC membrane to CSPG molecules also raises the possibility of close molecular interactions that are indicative of receptor-ligand pairs.

4.3. DRG CD44 upregulation and colocalization

Various studies have demonstrated a functional link between CD44 expression and cell behavior in the presence of proteoglycans for several cell types. For example, CD44 expression in motor neurons is upregulated after spinal chord injury in parallel with increased CSPG expression [13,35]. Additionally, CD44 expression in retinal ganglion cells and ECM CSPG expression have both been shown to be required for normal RGC pathfinding in the optic chiasm during development [7,36,37]. Compelling evidence has also been presented that leukocyte rolling on vascular endothelial cells is mediated in part by CD44 – chondroitin sulfate interactions [15]. In vitro studies of molecular interactions have demonstrated that CD44 binds specifically to AG and to other CSPGs [14–16].

CD44 upregulation in response to AG observed in this study provides new information on the relationship between proteoglycans and CD44 expression in neurons. Although in vivo CD44 upregulation has been observed in response to CNS trauma [13], it has remained unclear what contribution trauma-induced local ECM proteoglycan expression had on this outcome. With the ability to control the presence and density of AG in outgrowth lanes, we were able to separate the effects of neuronal trauma and ECM presentation. As a result, we found that although all neurons in this study experienced axotomy during explantation, the presence of AG in outgrowth pathways triggered increased CD44 expression vs. DRGs grown on LN only substrata. These results suggest that ECM signals and DRG proteoglycan sensing more robustly affect CD44 regulation in neurons than cell trauma. It is also significant that neurons growing on lanes *not* containing AG clusters also significantly upregulated CD44 expression compared to LN only controls. This observation suggests that even brief filopodia and GC edge sampling of AG boundaries is sufficient to trigger the CD44 expression response. CD44 upregulation in low-density AG outgrowth lanes also demonstrates the sensitivity of DRG response to proteoglycans.

Colocalization of CD44 molecules over AG clusters as seen in Fig. 5, panels B&C adds to other evidence that DRG sensing of CSPGs occurs through specific receptor-ligand interactions. The observation also suggests that an active transport mechanism controls CD44 spatial distribution in GCs. However, to bolster evidence of CD44 as a proteoglycan receptor, further experimentation will be required to functionally link neuronal behavior to CSPG-CD44 interactions. For example, selective labeling of only membrane bound CD44 molecules could more conclusively reveal CD44 colocalized expression with AG clusters. Additionally, monoclonal antibody binding to CD44 during outgrowth assays could reveal if blocking presumed CD44-CSPG interactions abolishes or diminishes observed inhibitory effects of CSPGs on neuron pathfinding. TIRF imaging of fluorescently tagged CD44 molecules could also reveal if membrane bound CD44 preferentially associates with substratum AG molecules.

5. Conclusion

In summary, the neuron morphology and CD44 expression results demonstrate the exquisite sensing and discriminating capacity of growth cone structures in response to near single molecule proteoglycan encounters. By combining precise placement of proteoglycan clusters through micropatterning and TIRF imaging, we were able to observe the three-dimensional morphology of DRG substratum contacts on various spatial arrangements of AG molecules. These observations led to the finding that CSPGs exert a repulsive effect, possibly in part by an anti-adhesive mechanism, on filopodia structures, GC, and cell body membranes. We demonstrated that CD44 expression in DRGs is influenced by the presence and relative abundance of substratum bound AG molecules, indicating a causal link between CSPG expression and CD44 neuronal upregulation in CNS injuries. Together, our results provide new insights into the molecular mechanisms of proteoglycan inhibition, and demonstrate how neurons sense and respond to CSPGs during developmental or regenerative pathfinding through well defined cues and fields of diffuse or dense CSPG expression.

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Fig. 1.

Transfer of adsorbed AG molecules from PDMS stamps resulted in the reproduction of the stamp relief patterns onto growth substrata. (A) Atomic force microscopy image of PDMS stamp. Image depicts one of five periodic lanes each containing a unique pillar density. Highest points shown as the lightest areas are transfer points of AG. (B) Fluorescence images of micropatterned AG layers on LN-coated glass substrata. Patterns consisted of repeating 30 μm wide lanes containing 0, 2, 4, 7, 11, or 31% AG density with 100 μm wide “100%” AG fields separating each lane. AG fluorescence shown as lightest area intensity. Dark areas are exposed LN layer. Scale bar = 10 μm . (C–E) Fluorescence images of adsorbed or stamped AG molecules on LN-coated glass substrata. Single AG molecules, at 400 nm in length, are resolvable in the images. (C) AG molecules adsorbed at a density of 136 ng/cm^2 onto LN layers. (D) Stamped AG molecules were transferred at a density of 248 ng/cm^2 onto LN-coated glass. (E) Stamped AG clusters in the 31% density lane were transferred at an average lane density of 77 ng/cm^2 , with local cluster density at 158 ng/cm^2 . Scale bars (C–E) = 2 μm .

Fig. 2.

DRG neurite orientation and branching were directed by 30 μm -wide lanes, while filopodia outgrowth was affected at the AG cluster level. (A–C) Composite fluorescence images of actin-stained (green) DRG growth cone and neurite structures on mixed AG (red) and LN (black) patterns. (A) Small growth cone (GC) with extending filopodia on a 31% density AG cluster pattern. The growth cone spans multiple AG clusters while filopodia grow mainly between AG molecules on LN sites. Scale bar = 10 μm . (B) Large growth cone edge on 31% AG pattern exhibiting filopodial discrimination of AG and LN sites. Scale bar = 5 μm . (C) Despite overall neurite orientation being directed by LN lanes, neurites indiscriminately grew over AG clusters (Arrows), demonstrating no bias for LN sites. Scale bar = 20 μm . (D) Edge enhanced image of growth cone and neurites on AG pattern. Branching neurite angle measurements are shown in red while neurite segment angle measurements with respect to the LN lane edge are shown in green. In general, multiple segment and branching angles were measured for each growth cone extension. (E) Filopodial organization exhibited significant bias to LN site adhesion for three of five cluster densities with no apparent AG density effect. * $p < 0.04$ paired 2-tail t-test vs. no bias cases. n = number of imaged growth cone edges. (F) Straight neurite segment orientation between turning and branching points was significantly biased to outgrowth lane alignment but was not affected by AG cluster alignment or density. $p < 0.001$ ANOVA for all cases, * $p < 0.001$ vs. LN Control by t-test. n = number of imaged neurite segments. (G) Neurite branching angles were significantly reduced on five of six outgrowth lanes compared to neurites on non-patterned LN Control substrates, suggesting branching outgrowth direction was constrained by lane boundaries. $p < 0.001$ ANOVA for all cases, * $p < 0.012$ vs. LN Control by t-test. n = number of imaged neurite branching points. ND – insufficient data. Data are mean values $\pm$ 95% confidence intervals.

Fig. 3.

Growth cones navigated mixed protein fields through a process of filopodial sampling of LN and AG sites and initial extension by interacting with LN sites. Time-lapse sequence of outgrowth on an 11% density AG cluster pattern, presented as a set of phase and fluorescence composite images. Arrows pointing down to the left indicate the tip of the leading filopodia in each image. Growth cone forward movement in general followed the AG cluster directions. Arrows pointing in the up direction indicate the location of the growth cone leading edge as it navigated between AG clusters. Note the growth cone is a narrow and extended flattened structure as typically observed on homogeneous LN surfaces. Scale bar = 10 μ m.

Fig. 4.

TIRF imaging revealed close association of DRG cell bodies and growth cones with the substratum, while neurites were less often attached to the substratum. Growth cone and cell body membranes exhibited numerous close contacts with LN sites while membranes arched over adjacent AG clusters. (A) Montage image of DRG outgrowth on a 31% density AG cluster pattern; left panel – epifluorescence image of AG pattern (red), on LN background (black); middle panel – epifluorescence image of DRG (green); right panel – TIRF image of DRG neurites and growth cone with enlarged inset on bottom left demonstrating areas of growth cone membrane-substratum separation over AG clusters. Arrows in the TIRF image indicate portions of neurites that are not in the TIRF excitation field due to increased separation from the substratum. Scale bar = 20 μm. (B) Montage of DRG cell body on 31% density AG cluster pattern; left panel – epifluorescence image of AG pattern (red) on LN background (black). DRG cell body is also visible because of cytoplasmic dye red spectral emission; right panel – TIRF image of DRG cell body (CB) and extended neurites terminating in growth cones (GC). Scale bar = 20 μm. (C) TIRF image of DRG cell body enlarged and scaled in intensity from

image (B). Cell body exhibits hexagonal pattern of membrane bending over AG clusters. Inset on bottom left – enlarged image of membrane separations over AG clusters. Scale bar = 10 μm . (D) TIRF image of a growth cone exhibiting membrane bending over a 7% density AG cluster pattern. Scale bar = 20 μm .

Fig. 5. DRG growth cone expression of the cell adhesion molecule CD44 was upregulated over time and in an AG substratum density dependent fashion. CD44 (green) spatial expression colocalized with AG (red) clusters suggesting CD44 interactions with PGs. (A) Fluorescence composite image of a CD44 labeled growth cone spanning multiple AG clusters on an 11% density pattern on LN background (black). Scale bar = 20 μ m. (B) Montage fluorescence image of DRG cell body on 31% density AG cluster pattern; left panel – composite image of cell body and AG pattern exhibiting CD44 co-localization with AG clusters; right panel – cell body image with CD44 clusters visible in bottom lamellapodia structure and in budding neurites on left and right (Arrows). Scale bar = 20 μ m. (C) Montage of portion of lamellapodia enlarged

from image (B); left panel – CD44 clusters in hexagonal pattern; middle panel – CD44 clusters from left panel are outlined to emphasize hexagonal organization; right panel – composite of colocalized CD44 clusters and underlying AG clusters; Scale bar = 5 μm . (D) Relative CD44 expression density in DRG growth cones vs. post-seeding time and outgrowth substratum condition. CD44 expression was significantly upregulated in a dose and time dependent manner vs. the LN control case. ANOVA identified significant differences between cases at 9 and 15 hour outgrowth times, $p < 0.006$ for 9 hrs, $p < 0.087$ for 12 hrs, $p < 0.001$ for 15 hrs. * p values for 15 hrs found by t-test vs. LN Control. (E) Number of growth cones imaged for each case in (D). Data are mean values $\pm$ 95% confidence intervals.

Aggrecan Surface Pattern Characterization
Table 1

Expected % AG density	Avg AG density [ng/cm ²]	Avg AG molecules per cluster	Avg single cluster area [μm ²]	Actual % AG density	Notation in text
0	0	-	-	0	0%
6	3.74±0.59	1.4	0.51	1.51	2%
12.5	8.86±1.24	1.6	0.52	3.57	4%
25	16.2±1.80	1.4	0.51	6.53	7%
37.5	27.0±2.90	1.5	0.53	10.9	11%
50	77.2±7.40	3.4	0.89	31.1	31%
100	248±11.0	-	-	100	100%

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