Spatial distribution affects the role of CSPGs in nerve regeneration via the actin filament-mediated pathway

Jian-Long Zou,1*, Jia-Hui Sun,2 Shuai Qiu,1 Shi-Hao Chen,1 Fu-Lin He1, Jia-Chun Li,1 Hai-Quan Mao,1, Xiao-Lin Liu1,2, Da-Ping Quan2, Yuan-Shan Zeng3, Qing-Tang Zhu1,2,*

1 Department of Microsurgery, Orthopedic Trauma and Hand Surgery, The First Affiliated Hospital, Sun Yat-sen University, No. 58, Zhongshan 2nd Road, Guangzhou 510080, China
2 Guangdong Provincial Peripheral Nerve Tissue-engineering and Technology Research Center, Guangzhou 510080, China
3 Key Laboratory for Stem Cells and Tissue Engineering, Sun Yat-sen University, Ministry of Education, Guangzhou, Guangdong 510080, China
4 PCFM Lab, GD HPPC Lab, School of Chemistry, Sun Yat-sen University, Guangzhou, Guangdong 510127, China
5 Orthopedics Department, The Third Affiliated Hospital, Sun Yat-sen University, 600 Tianhe Road, Guangzhou, Guangdong 510630, China
6 Institute for NanoBioTechnology, Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA

ARTICLE INFO

Keywords:
CSPGs
Spatial distribution
Axonal regeneration
Growth cone
Jasplakinolide

ABSTRACT

CSPGs are components of the extracellular matrix in the nervous system, where they serve as cues for axon guidance during development. After a peripheral nerve injury, CSPGs switch roles and become axon inhibitors and become diffusely distributed at the injury site. To investigate whether the spatial distribution of CSPGs affects their role, we combined in vitro DRG cultures with CSPG stripe or coverage assays to simulate the effect of a patterned substrate or dispersive distribution of CSPGs on growing neurites. We observed neurite steering at linear CSPG interfaces and neurite inhibition when diffused CSPGs covered the distal but not the proximal segment of the neurite. The repellent and inhibitory effects of CSPGs on neurite outgrowth were associated with the disappearance of focal actin filaments on growth cones. The application of an actin polymerization inducer, jasplakinolide, allowed neurites to break through the CSPG boundary and grow on CSPG-coated surfaces. The results of our study collectively reveal a novel mechanism that explains how the spatial distribution of CSPGs determines whether they act as a cue for axon guidance or as an axon-inhibiting factor. Increasing our understanding of this issue may promote the development of novel therapeutic strategies that regulate the spatial distributions of CSPGs to use them as an axon guidance cue.

1. Introduction

Chondroitin sulfate proteoglycans (CSPGs) are composed of a protein core and chondroitin sulfate (CS) side chains (Avram et al., 2014). Extensive evidence shows that CSPGs are major inhibitors of neurite growth in the nervous system and that they mainly accumulate in the scar and perineuronal nets of the central nervous system (CNS) or in the endoneurial and epineurial regions of the peripheral nervous system (PNS) (Massey et al., 2006; Muir, 2010). Under physiological conditions, CSPGs in the embryonic CNS create a chemical barrier that can drive neuronal migration or guide the direction of axonal growth by exerting a neurite-inhibiting effect to adjust the trajectory of nerve fibers, thereby guiding them to their appropriate targets during nerve development or, alternatively, inhibiting neurons or neurites from entering inappropriate territories (Beller et al., 2013). In this well-compartmentalized manner, CSPGs can also stabilize nerve structures by inhibiting spontaneous axonal branching (Masuda et al., 2004; Pizzorusso et al., 2002), and combined with other ECM molecules, such as laminin (LN), fibronectin (FN) and collagen, they participate in the formation of myelin sheaths (Muir, 2010).

When an injury occurs in the CNS, reactive astrocytes produce more CSPGs diffusely around the lesion site (McKeon et al., 1999), and this increase in the protein level of CSPGs is involved in the formation of glial scars, which present a physical and chemical barrier that inhibits neurite regeneration and deters the functional recovery of denervated organs (Silver and Miller, 2004). A similar pathological process also occurs in peripheral nerve injuries. Many previous studies have demonstrated that after a peripheral nerve injury, CSPG levels increase and exhibit an irregular spatial distribution in the distal nerve stump (Gause et al., 2014; Graham and Muir, 2016).

* Corresponding author at: Department of Microsurgery, Orthopedic Trauma and Hand Surgery, The First Affiliated Hospital, Sun Yat-sen University, No. 58, Zhongshan 2nd Road, Guangzhou 510080, China.
E-mail address: zhuqingt@mail.sysu.edu.cn (Q.-T. Zhu).

https://doi.org/10.1016/j.expneurol.2018.05.023
Received 12 January 2018; Received in revised form 24 May 2018; Accepted 27 May 2018
Available online 29 May 2018
0014-4886/ © 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/BY/4.0/).
Chondroitinase ABC (ChABC) is an enzyme that catalyzes the removal of the chondroitin sulfate and dermatan sulfate side chains of proteoglycans; is therefore used to degrade CSPGs. Studies have used ChABC to treat animals with spinal cord or peripheral nerve injuries, and this treatment improved functional recovery (Graham et al., 2007). However, other studies have reported that the removal of CSPGs by ChABC increases non-directional axon growth, thereby offsetting some benefits of increased nerve regeneration (English, 2005; de Ruiter et al., 2008). This indicates that CSPGs are required for orderly nerve regeneration.

By enhancing the adsorption of CSPGs at the surface of polymer membranes, Snow et al., and Man et al. created a stable CSPG boundary on a poly-L-lysine-coated substrate, and successfully oriented neurite outgrowth by selectively inhibiting neurite growth in the CSPG-bounded region (Snow et al., 1990; Man et al., 2014). Considering the wide variety of CSPG spatial patterns that can occur in normal physiology, after injury and following pharmaceutical intervention, the effects of different CSPG distribution patterns on neurite growth remain to be further defined.

We hypothesized that the role of CSPGs in nerve regeneration could be modulated by altering the spatial distribution of CSPGs. In this study, we developed a series of CSPG spatial distribution assays based on a rat dorsal root ganglion (DRG) culture model. A CSPG boundary was created by placing CSPG-soaked cellulose strips on a poly-α-lysine-coated surface and, thereby, the orientation effect of a CSPG boundary on growing neurites was investigated. A compartmentalized CSPG cover assay was developed by mixing CSPGs with hydrogel then covering the proximal and distal neurites respectively. Based on these models, the effects of CSPGs on neurite retraction and axonal branching were investigated. In addition, we discovered that the local redistribution of actin filaments mediated changes in neurite morphology, which resulted in the steering/retraction of neurite or inhibited axonal branching induced by CSPGs. Furthermore, stabilization of actin filaments with jasplakinolide promoted the ability of neurites to cross CSPG-enriched zones.

2. Materials and methods

2.1. DRG neurons culture

DRGs dissected from P1 SD rats were incubated with 0.25% trypsin at 37 °C for 15 min, then dissociated by pipetting up and down 200 times. 10% FBS was used to inactivate the trypsin. The cells were pelleted by centrifuging at 800 rpm for 2 min. The supernatant was then discarded and the cells were resuspended in Neurobasal media containing 2% B27, 0.3% l-glutamine, 100 ng/mL NGF and 1% Penicillin-Streptomycin Solution. Cells in suspension were seeded on poly-α-lysine-coated 24 well dishes at a density of 1.0 × 10⁵ cells/well and incubated at 37 °C for 48 h, followed by CSPG boundary or CSPG cover assays.

2.2. DRGs ex vivo culture

DRGs were dissected from P1 SD rats and collected in cold DMEM/F12 medium. The redundant roots of the DRGs were removed under a stereomicroscope, and 10 whole dorsal root ganglia were seeded in each vehicle pre-coated (poly-α-lysine + vehicle strip) or CSPG pre-coated (poly-α-lysine + CSPGs strip) well. The DRGs were then incubated in neurobasal media (Gibco; Grand Island, NY) containing 2% B27, 0.3% l-glutamine (Gibco; Grand Island, NY), and 100 ng/mL nerve growth factor (NGF) (Peprotech; Rocky Hill, NJ) in a 37 °C incubator with 5% CO₂ and 92% humidity. The medium was changed every other day. All steps were performed under sterile conditions. After the DRGs were incubated for 48–72 h, they were firmly attached to the bottoms of the culture dishes and had radially extended 1–2 mm neurites. A subset of DRGs in the vehicle group were taken out of the incubator, and the culture medium in these wells was gently removed to prepare for the CSPG cover assay.

2.3. Preparation of the CSPG boundary

This method was modified from a previous publication (Snow et al., 2001). Briefly, the bottoms of six-well dishes (Corning; Corning, NY) were covered with 0.01% (w/v) poly-α-lysine solution (PDL, Sigma-Aldrich; St. Louis, MO) for 2 h and then washed twice with PBS and air-dried at RT for 1 h to provide an adhesive substratum. CSPGs (10 µg/mL; Millipore; Temecula, CA) were applied by placing cellulose strips on the poly-α-lysine-coated surface until dry (15 min). The cellulose strips were then removed, and the wells were washed with PBS to remove unbound CSPGs. The resulting CSPG boundaries were confirmed by adding a fluorescent Alexa Fluor 555-reactive dye (Molecular Probes; Eugene, OR) to the CSPGs solution (Fig. 1A).

2.4. CSPG cover assay

Neurobasal medium containing 0.5% (w/w) genipin (Sigma-Aldrich; St. Louis, MO), and 50% (v/v) Growth factor-reduced Matrigel (Corning; Corning, NY) was prepared as a pre-gel solution. The CSPGs were dissolved in the pre-gel solution to reach a concentration of 10 µg/mL (all steps above were performed on ice). After it was thoroughly mixed, the CSPG pre-gel solution was dripped onto the surface of 12 mm cover slips and allowed to gel in a 37 °C incubator for 10 min. We then rapidly covered DRGs with the gel pad side down. Some DRGs had only the distal part of their neurites covered, while others were completely covered except for the distal segments of their neurites. Neurobasal medium was added, and the DRGs were further incubated for 48 h.
2.5. Immunoﬂuorescence staining

The culture medium and cover slides were removed, and the DRGs were washed twice with cold PBS and then ﬁxed with 4% paraformaldehyde (PFA) for 2 h. After they were washed with PBS, the samples were penetrated and blocked with PBS containing 0.3% (v/v) Triton X-100 and 10% (v/v) goat serum for 30 min. They were then incubated with rabbit anti-neuroﬁlament 200 (NF200) antibodies (Sigma-Aldrich; St. Louis, MO) to label the neurites or mouse anti-α-tubulin antibodies (Sigma-Aldrich; St. Louis, MO) to label microtubules. The slides were then labeled with the corresponding Alexa 488- or Alexa 555-conjugated secondary antibodies (Abcam; Cambridge, MA). After they were washed with PBS, the DRGs were incubated with PBS containing 0.3% (v/v) phalloidin-iFluor 555 conjugates (AAT Bioquest; Sunnyvale, CA) for 45 min at room temperature to visualize the actin ﬁlaments. For Protein tyrosine phosphatase σ (PTPσ) receptor staining, goat anti-PTPσ antibodies (Sigma-Aldrich; St. Louis, MO) and Alexa 488-conjugated donkey anti-goat secondary antibodies (Invitrogen; Carlsbad, CA) were used. The samples were mounted with Fluoroshield containing DAPI (GeneTex; Irvine, CA) to stain the cell nuclei. The resulting ﬂuorescence was imaged with a Carl Zeiss ﬂuorescence microscope.

2.6. Analysis of neurite turning, branching and fragmentation

DRGs grown in CSPG strip-patterned dishes were imaged under a 40 × objective lens along the CSPGs boundary. We imaged two predeﬁned ﬁelds in each well. Neurites were counted and turning angles were measured using ImageJ software (National Institutes of Health; Bethesda, MD). The neurites that grew against the CSPG boundary and did not turn but stopped near the boundary were deﬁned as stopped neurites. The ratio of neurites that performed obvious turning was calculated, and the distribution of turning angles was analyzed. For neurite branching analysis, the region of interest (ROI) was deﬁned as a square with length of 200 μm and set at 1 mm distance from DRG tissue block. Within ROI, neurites with at least one branch were counted and marked as ﬁrst order branches. Neurites with second or higher order branches were counted and marked as second order branches. The number of neurites with ﬁrst order or second order branches were divided by the number of total neurites, then multiplied by 100 to obtain the percentage of neurites with ﬁrst order or second order branches. Five ROIs were calculated for each individual experiment. To obtain the ratio of fragmented neurites at the proximal segment, we imaged ﬁve predeﬁned ﬁelds at a 0.5 mm distance from DRG tissue block under a 40 × objective lens and the number of neurites with fragmented structure was manually counted and divided by the number of total neurites in the ﬁeld. We also calculated the ratio of fragmented neurites at the distal segment from images of distal neurites.

2.7. Neurite extension distance and neurite density calculations

At 48 h after the CSPG cover assays were begun, images of the DRGs were acquired before the cover slides were removed. The brim of each cover slide was indicated with an arced dotted line. We also imaged DRGs with extended neurites that were processed for immunoﬂuorescence staining of NF200. In some of the experiments, the neurites were divided into covered and uncovered subgroups depending on whether the distal end of the neurite was covered by hydrogel (Fig. 4). The neurite extension distance was measured from the rim of the DRG tissue block to the distal point of the neurite. The DRG tissue block was deﬁned as the center, and multiple measurements were performed every π/12 rad to obtain an average value. To calculate neurite density, circles with a predeﬁned radius were drawn along the neurites (DRG block as the center too). The number of neurites that passed through each unit radian was counted as the neurite density at a speciﬁc distance from the rim of the DRG tissue block. Additionally, multiple measurements were obtained every π/12 rad to determine the average value.

2.8. Morphological analysis of growth cones

This method was modiﬁed from previous publications (Williams et al., 2002; Smirnov et al., 2014). In a subset of DRG culture experiments, images of growth cones were captured under a 63 × objective lens. The lengths of ﬁlopodia were measured directly in Leica Application Suite (LAS). Growth cone perimeters were obtained by tracing growth cone out-lines, and the area was computed using ImageJ software.

2.9. Statistical analysis

All data were analyzed using SPSS version 19.0 (IBM; Armonk, NY). We compared continuous variables between two groups using Student’s t-test (two-tailed) if the data were normally distributed or the Wilcoxon Mann-Whitney test when normality was rejected. The difference among more than two groups was determined using one-way ANOVA followed by LSD post hoc multiple comparison tests. Corrected p values < 0.05 indicated a statistical signiﬁcance.

3. Results

3.1. CSPG boundary leads to neurite turning

To assess the effect of CSPGs on neurite orientation, we created a smooth CSPG boundary by applying a solution containing CSPGs (10 μg/mL) to a PDL-coated substrate (Fig. 1A). The absence of neurite outgrowth in the PDL + CSPG-coated area was compared to the abundance of neurites in the PDL only or PDL + vehicle-coated area to conﬁrm the neurite-inhibiting effect of the CSPGs. The visible interface consisted of neurites aligned between neurite-absent and neurite-abundant regions (arrowhead) that further conformed to the retained CSPG boundary during the cell culture process (Fig. 1B1, B2). The angle at which the neurites turned was measured in cultures stained to visualize neuroﬁlaments (Fig. 1C). We calculated the proportions of neurites that displayed different turning angles in the region closest to the CSPG boundary. The results showed that there was a signiﬁcantly higher proportion of angular neurites (0.88 ± 0.09) than stagnate neurites that did not turn (0.12 ± 0.09, n = 4 sets of cultures, 40 neurites were counted in total, p < 0.01, Fig. 1D). A distribution analysis of turning angles further revealed that neurites with a 0–30° turning angle (0.42 ± 0.12) and neurites with a 30–60° turning angle (0.35 ± 0.08) were present in larger proportions than neurites with 60–90° turning angle or stopped neurites without a turning angle (0.12 ± 0.08 and 0.12 ± 0.09, respectively, n = 4 sets of cultures, 40 neurites were counted in total, p < 0.01, Fig. 1E). These data suggest that a smooth CSPG interface can guide neurites to turn and is more likely to result in a small turning angle.

3.2. Coverage with CSPGs leads to inhibited neurite outgrowth and decreased neurite density

We next examined the effect of CSPG coverage on neurite outgrowth behavior using a DRG ex vivo culture model. At 48–72 h after incubation was begun, DRGs with extended neurites were covered on each side with either a hydrogel containing CSPGs or a hydrogel containing vehicle (Fig. 2A, Fig. S2). The DRGs were then incubated for another 24 h. To verify that the axon-like structures observed under a light microscope were neurites, we performed immunofluorescence staining with a neurite-speciﬁc protein marker (NF200), which demonstrated that the axon-like structures were perfectly marked by the NF200 signal (Fig. 2B1, B2). The full scope images of DRGs exhibited neurite outgrowth in different regions. Clearly shorter neurites were observed in
The CSPG-coverage treatment inhibited neurite growth and axonal branching. A Schematic of DRG neurites covered with CSPG and vehicle gel pads. B1–B2 Images of neurites observed under phase contrast (pH) microscopy were overlapped with NF200 fluorescence signals. C The growing status of neurites in the CSPG-covered, vehicle-covered and gel uncovered areas. D Histogram showing a comparison of neurite lengths among different groups. The lengths of the neurites were significantly shorter in the CSPG-covered area than in the vehicle-covered area or gel uncovered areas. E A distance-dependent line chart showing that neurite density was lower in the CSPG-covered area than in the vehicle-covered or gel uncovered areas. As the distance increased, the neurite density in the CSPG-covered area significantly decreased. F1–F2. Branched neurites at 1 mm away from the DRG tissue block in CSPG-covered area and vehicle-covered area. G. Neurites with the first order and second order branches were significantly lower in the CSPG-covered area than that in the vehicle-covered area. *p < 0.01, Scale Bar = 20 μm in B1 and B2, 1 mm in C.

Fig. 2. The CSPG-coverage treatment inhibited neurite growth and axonal branching. A Schematic of DRG neurites covered with CSPG and vehicle gel pads. B1–B2 Images of neurites observed under phase contrast (pH) microscopy were overlapped with NF200 fluorescence signals. C The growing status of neurites in the CSPG-covered, vehicle-covered and gel uncovered areas. D Histogram showing a comparison of neurite lengths among different groups. The lengths of the neurites were significantly shorter in the CSPG-covered area than in the vehicle-covered area or gel uncovered areas. E A distance-dependent line chart showing that neurite density was lower in the CSPG-covered area than in the vehicle-covered or gel uncovered areas. As the distance increased, the neurite density in the CSPG-covered area significantly decreased. F1–F2. Branched neurites at 1 mm away from the DRG tissue block in CSPG-covered area and vehicle-covered area. G. Neurites with the first order and second order branches were significantly lower in the CSPG-covered area than that in the vehicle-covered area. *p < 0.01, Scale Bar = 20 μm in B1 and B2, 1 mm in C.

The statistical analysis of measurements of neurite length further confirmed that neurite outgrowth lengths were > 30% shorter in CSPG-covered areas (1.67 ± 0.32 mm) than in vehicle or gel uncovered regions (2.48 ± 0.20 mm and 2.42 ± 0.26 mm, respectively; n = 6 wells, *p < 0.01, Fig. 2D). By counting the number of neurites that passed through each unit radian, we calculated the average density of the neurites at 0.5 mm, 1 mm and 1.5 mm away from the DRG tissue block. A distance-dependent line chart showed that the neurite density was lower in the CSPG-covered area than in the vehicle-covered area regardless of the distance (n = 7 DRGs, *p < 0.01). Theoretically, the neurites that grow radially out of a DRG tissue block vary in length so that as the distance away from the DRG increases, neurite density also decreases. However, in our experiments, neurite density slightly increased in the vehicle and gel uncovered areas as the distance increased, suggesting that axonal branching occurred during the process of neurite outgrowth. However, the neurite density in the CSPG-covered area decreased dramatically as the distance from the DRG block increased (Fig. 2E). By counting the neurites with branches in CSPG-covered areas (Fig. 2F1) and vehicle-covered areas (Fig. 2F2), the percentage of neurites with first order branches and second order branches were calculated at 1 mm away from the DRG tissue block. Results showed that the percentage of branched neurites was significantly lower in the CSPG-covered area (28.2 ± 6.8% and 9.6 ± 4.7% of the neurites with first order branches and second order branches respectively) compared with that in the vehicle-covered area (90.3 ± 7.9% and 73.4 ± 12.2% of the neurites with first order branches and second order branches respectively, n = 6 areas, p < 0.01, Fig. 2G). These results suggest that axonal branching events were inhibited.

The reason that the neurite lengths were shorter in the CSPG-covered area may be that neurite outgrowth speed was slower or because the neurites had retracted. To resolve this issue, we tracked the dynamic changes in neurite length that occurred under CSPG coverage. At 48 h after the incubation was begun, significant retraction was detected in the distal segments of the neurites (Fig. 3A1, A2). A time-dependent line chart revealed that > 31% of the CSPG-covered neurites retracted, whereas 98% of the vehicle-covered neurites elongated (Fig. 3B). NF200 immunofluorescence staining showed that there was good structural continuity in both the proximal and distal neurites in the vehicle-covered area. In contrast, coverage by CSPGs caused serious structural disintegration in the distal but not the proximal parts of the neurites. E Histogram showed the ratio of fragmented neurites at proximal and distal segment, between vehicle and CSPG-covered areas. *p < 0.01, Scale Bar = 50 μm in A1–A2, 10 μm in C1–D2.
3.3. CSPG coverage of neuronal cell bodies and proximal neurites had no inhibitory effect on distal neurite outgrowth

Vehicle-covered area (n = 5 areas, > 50 neurites counted in each area, p < 0.01 Fig. 3E).

Together, these results demonstrate that when neurites are in all-around contact with CSPGs, the CSPGs inhibit both neurite outgrowth and branching, and the distal segments of the neurites are more vulnerable than the proximal segments to CSPG-induced structural disintegration.

3.3. CSPG coverage of neuronal cell bodies and proximal neurites had no inhibitory effect on distal neurite outgrowth

To study neurite outgrowth behavior when CSPGs can act only on the neuronal soma and proximal neurites, DRG tissue blocks were cultured for 48 h and then covered with A CSPG-containing hydrogel or B vehicle-containing hydrogel for another 48 h. C In the gel uncovered group, the neurites extended a consistent distance in all directions. D Distal coverage by CSPGs significantly inhibited the neurite extension distance, while coverage at the proximal neurites and DRG soma did not inhibit growth by the distal neurite. E Neurite density significantly decreased in the CSPG-covered area, while the density of distal neurites outside the CSPG-covered area was not significantly different from that observed in the vehicle group. F Statistical analysis showed that axon branches were significantly decreased in CSPGs covered area but not in CSPGs uncovered area compared with gel uncovered area. * p < 0.05, ** p < 0.01, scale bar = 1 mm in A–C.

areas in the CSPG-treated group than in the gel uncovered group (p < 0.05, Fig. 4E). We manually quantified percentages of neurites that contained first order and second order branches. Under the CSPGs covered area, the percentage of first order branches (21.58 ± 6.84%) and second order branches (8.07 ± 3.68%) was significantly lower than that in CSPGs uncovered area (90.53 ± 9.30% of first order branches and 70.01 ± 9.82% of second order branches) and gel uncovered area (92.81 ± 7.02% of first order branches and 74.91 ± 8.77% of second order branches, n = 5 DRGs, p < 0.01, Fig. 4F).

To delineate expression and distribution of the CSPG receptor in DRG neuron, we performed immunostaining of pTPR, one major receptor of CSPGs, in our DRG culture model. pTPR was evenly distributed in a punctate pattern in axons and growth cones in the vehicle group (Fig. 5A4, B). However, it becomes concentrated in dystrophic growth cones after treatment with CSPGs (Fig. 5C, D). pTPR fluorescence intensity was significantly higher in the growth cone of CSPG-treated cells than that in the soma or axon (Fig. 5E).

These data collectively demonstrate that the neuronal response to CSPGs heavily relies on the distribution of pTPR and only distal neurites are responsive to the inhibitory effect of CSPGs.

3.4. Disappearance of actin filaments on distal neurites after CSPG treatment

Actin filament bundles are required for growth cones to avoid inhibitory guidance cues during turning (Challacombe et al., 1996). Hence, actin dynamics are expected to mediate the effects of CSPGs on neurite motility.

We stained F-actin with Cy3-conjugated phalloidin and labeled DRG neurites with an antibody against NF200 (Fig. 5A1–B2). In vehicle-containing hydrogel-covered neurites, the F-actin signals masked NF200 signals and were more concentrated at the peripheral region of the growth cones (arrowhead, Fig. 5A1). In contrast, CSPG coverage led to complete disappearance of F-actin in the growth cones (arrow, Fig. 5B1). A similar phenomenon was also observed in CSPG-covered neurites that were double-stained with Cy3-conjugated phalloidin and α-tubulin antibodies. Such double staining showed that the F-actin signal was completely lost in growth cones and distal neurites, whereas the change in α-tubulin signals was relatively subtle (Fig. 5C, D). We manually counted and computed the ratio of growth cones with F-actin signals and found that there were significantly fewer F-actin-positive growth cones in CSPG-covered neurites (3.54 ± 3.35%) than in vehicle-covered neurites (95.12 ± 4.09%; n = 5 wells, > 100 neurites counted in each group, p < 0.01, Fig. 5E). The length of filopodia and the areas of growth cone were also measured (Fig. 5F1–G2). Significant reduction of filopodial length and growth cone area were detected in CSPGs covered neurites (0.39 ± 0.26 μm and 5.91 ± 2.28 μm² respectively), compared with that in vehicle covered neurites (5.26 ± 1.97 μm and 38.69 ± 8.04 μm² respectively, n = 10 neurites, p < 0.01). These data demonstrate that neurite actin filament dynamics were more sensitive than intermediate filaments (labeled with NF200) and microtubules (labeled with α-tubulin) to the inhibitory effects of CSPGs.

3.5. The actin filament stabilizer jasplakinolide attenuates the neurite-inhibiting and orienting effect of CSPGs

The disappearance of actin filaments in the growth cone may result from increased actin depolymerization and/or decreased actin polymerization. To investigate the causal link between the effects of CSPGs on actin filament motility and neurite retraction and steering movements, we added jasplakinolide (JAS), a cell-permeable molecule to promote actin polymerization and prevent actin depolymerization (Xu et al., 2017), to our CSPG-covered DRG cultures. JAS produced a dose-dependent response in reducing neurite lengths in DRG cultures with
mechanisms by which CSPGs in group, 92 neurites counted in vehicle group, 166 neurites counted in JAS involved altering the balance between actin and depolymerization.

Scale bar = 20 μm in B1–B3, 20 μm in C1–C2.

4. Discussion

CSPGs have been proposed to function as neurite guidance factors in the developed CNS, as an inhibitor of spontaneous axonal branching in the adult PNS, and as an inhibitor of neurite growth in injured nerve tissues (Beller et al., 2013; Masuda et al., 2004; Pizzorusso et al., 2002). However, the mechanisms that influence the role that CSPGs play in guiding the direction of neurite regrowth, regulating axonal branching and inhibiting neurite growth remain unknown. After a sciatic nerve crush injury, the area exhibiting inhibited axonal regeneration often displays a disordered spatial distribution of CSPGs (Fig. S1). We hypothesized that the effects of CSPGs on DRG neurite growth behaviors can differ based on their spatial distributions.

The ability to initiate the correct orientation of regenerated axons is one of the factors that contributes to improved nerve repair effects. Some in vitro studies have shown that adsorbing the CSPG boundary on a substrate layer can influence neurite guidance by prohibiting neurites from growing onto the CSPG-bounded substrate (Snow et al., 1990; Man et al., 2014). CSPGs for 24 h (Fig. 6B2), the addition of JAS (28 nM) to the culture medium prevented the CSPG-mediated disappearance of actin filaments that formed both lamellipodia and filopodia (Fig. 6B1). While these actin filaments disappeared after the DRGs were covered with CSPGs for 24 h (Fig. 6B2), the addition of JAS (28 nM) to the culture medium prevented the CSPG-mediated disappearance of actin filaments that formed both lamellipodia and filopodia (Fig. 6B1). The results of the CSPG boundary assay showed that fewer growing neurites broke through the CSPG boundary in the vehicle-treated group (Fig. 6C1) than in the JAS (28 nM)-treated group (Fig. 6C2). The ratio of neurites that crossed the CSPG boundary was significantly higher in the JAS group (0.66 ± 0.16) than in the vehicle group (0.12 ± 0.06; n = 4 cultures, 92 neurites counted in vehicle group, 166 neurites counted in JAS group, p < 0.01, Fig. 6D). These data suggest that one of the mechanisms by which CSPGs influence neurite steering and retraction involves altering the balance between actin filament polymerization and depolymerization.

complete neurite inhibition occurring at concentrations above 100 nM (Fig. 6A, n = 5 DRGs). High magnification images of distal neurites showed that the peripheral regions of growth cones were dominated by actin filaments that formed both lamellipodia and filopodia (Fig. 6B1). While these actin filaments disappeared after the DRGs were covered with CSPGs for 24 h (Fig. 6B2), the addition of JAS (28 nM) to the culture medium prevented the CSPG-mediated disappearance of actin filaments and led to swollen growth cones that exhibited excessive actin filament accumulation (Fig. 6B3). The results of the CSPG boundary assay showed that fewer growing neurites broke through the CSPG boundary in the vehicle-treated group (Fig. 6C1) than in the JAS (28 nM)-treated group (Fig. 6C2). The ratio of neurites that crossed the CSPG boundary was significantly higher in the JAS group (0.66 ± 0.16) than in the vehicle group (0.12 ± 0.06; n = 4 cultures, 92 neurites counted in vehicle group, 166 neurites counted in JAS group, p < 0.01, Fig. 6D). These data suggest that one of the mechanisms by which CSPGs influence neurite steering and retraction involves altering the balance between actin filament polymerization and depolymerization.

**Fig. 5.** Treatment with CSPGs caused disappearance in neurite actin filaments. A1–B2 DRG neurons were cultured for 48 h and then covered with a vehicle or CSPGs gel for 24 h. Immunofluorescence staining showing the distribution of NF200 and F-actin in DRG neurons. In the vehicle group, F-actin was expressed all over the cell and enriched in the end tips of growth cone, (arrowhead). CSPG coverage resulted in the disappearance of F-actin fluorescence signals at the distal segment of the neurite (arrow). C In DRG ex vivo cultures treated with vehicle coverage, F-actin signals were observed at the end tips of the neurites (arrowhead). D CSPG coverage caused the apparent disappearance of F-actin but not α-tubulin in neurites. E The ratio of F-actin positive growth cones was significantly lower in CSPG-treated neurites than in vehicle-treated neurites. F1–G2 Represent images showing the measurement of filopodia length and growth cone area. H Comparison of the filopodia length and I Growth cone area between CSPGs covered neurites and vehicle covered neurites. *p < 0.01, Scale bar = 20 μm in A1–B2, 50 μm in C–D.

**Fig. 6.** Treatment with JAS alleviated the neurite-inhibiting effect of CSPGs. A JAS produced a dose-dependent response in reducing neurites length in DRG cultures, complete neurite inhibition occurred at the concentration above 100 nM. DRG neurons grew for 48 h in vitro and were then treated with B1 vehicle or B2 CSPGs (20 μg/mL) or B3 a combination of CSPGs (20 μg/mL) and Jasplakinolide (JAS, 28 nM) for 8 h, arrowhead indicates the growth cone with excessive F-actin accumulation. C1 CSPG boundary assay showing neurites turning at the CSPG boundary (indicated by a dotted line) in the vehicle group. C2 JAS (28 nM) treatment led some neurites to grow across the CSPG boundary. D Histogram showing that the ratio of neurites that crossed the CSPG boundary was higher after JAS treatment than in the Vehicle-treated group, *p < 0.01. Scale bar = 5 μm in B1–B3, 20 μm in C1–C2.
injuries is the regulation of axonal branching or collateral sprouting. Many previous studies have shown that collateral sprouting plays a positive role in the recovery of motor functions after a nerve injury (Massey et al., 2006; Hagg, 2006; Hahn et al., 2006; Schweger et al., 1995), whereas excessive axonal branching of sensory nerves usually contributes to the development of pain and autonomic dysreflexia (López-Álvarez et al., 2015). To prevent these problems, some experimental treatment strategies, such as anti-NGF serum administration, have been developed to reduce sprouting in sensory neurons (Ro et al., 1999).

In this study, we propose that CSPGs located in the shear around a neurite inhibit axonal branching but not neurite extension. By loading and anchoring CSPGs in hydrogels, we could easily control the spatial distribution of the CSPGs by regulating the shape of the hydrogel in vitro. Indeed, our results revealed that CSPGs applied to neuronal cell bodies and proximal neurites (i.e., the CSPG-covered areas) do not inhibit distal neurite elongation but effectively inhibit axonal branching. This is in line with our immunostaining of the CSPG receptor showing concentrated PTPσ expression pattern when growing neurites touched a CSPG gradient (Lang et al., 2015). These findings above suggest novel treatment strategies that can reduce pathological axonal branching without inhibiting axon elongation.

It is logical to next ask how the spatial distribution of CSPGs induces the effect of CSPGs on neurite outgrowth behaviors. Studies have recently shown that the Nogo receptor family members NgR1 and NgR3 and PTPσ are the receptors for CSPGs and that inactivating these receptors abolishes the ability of CSPGs to inhibit neurite growth (Dickendesher et al., 2012; Shen et al., 2009). The binding of CSPGs to their receptors activates the Rho (Rho-associated kinase, ROCK) signaling pathway which directs the formation of a wide range of cytoskeletal structures, including the retrograde actin flow in the growth cone (Monnier et al., 2003; Zhang et al., 2003). As a substrate of ROCK, Myosin II also participates in the regulation of actin-bundle turnover in neuronal growth cones (Lin et al., 1997; Medeiros et al., 2006; Amano et al., 2010; Yu et al., 2012). Inhibition of the Rho/ROCK pathway by C3 or Y-27632, or inactivation of myosin II with blebbistatin allows growing neurites to cross onto CSPG-rich areas (Monnier et al., 2003; Yu et al., 2012). Also, application of Cytochalasin B to block actin filament formation in DRG neurons inhibited the ability of growth cone turning to avoid CSPGs (Challacombe et al., 1996). These results together indicate that the dynamic nature of actin filaments may be a common downstream activity which mediates varied responses of axons to different CSPG patterns.

In this study, we compared the dynamics of the responses of each cytoskeletal component (e.g., actin filaments, microtubules and intermediate filaments) to CSPGs treatment. We found that the reorganization of the actin cytoskeleton was the most dramatically altered process, which indicates that actin-based growth cone motility plays a major role in neurite guidance to avoid repulsive cues (Omotade et al., 2017). In CSPG boundary assays, when a growing neurite encountered the CSPG boundary, the actin filaments in filopodia and lamellipodia were clearly retracted on the side of the growth cone facing the CSPG boundary and redistributed to the side facing away from the boundary (Fig. S3). These data suggest that the retracted filopodia and lamellipodia on the CSPG boundary side had less traction than the normally functioning filopodia and lamellipodia on the side facing away from the boundary allowing more traction to guide neurite steering. These data are supported by the finding that myosin II mediates actin-bundle turnover in response to CSPG-induced neurite steering (Yu et al., 2012).

Based on this discovery, it is reasonable to speculate that when an entire growth cone is covered by CSPGs, all of the actin filament-containing structures in the growth cone will disintegrate, eventually leading to axonal retraction. This was also confirmed by our CSPG-coverage assays, the results of which revealed that actin filaments in the distal neurite disappeared in a retrograde manner and that the morphologies of the microtubules and intermediate filaments in the distal neurites changed simultaneously, but in a relatively milder manner. The drastically altered distribution of actin filaments observed in distal neurites may indicate the diagnostic significance of their distribution in neurodegeneration. Altogether, these results suggest that different spatial distributions of CSPGs have different effects on actin filament reorganization in neurites and can eventually cause neurites to either turn or retract.

We also provided evidence showing that the application of a cell-permeable actin polymerization and stabilization inducer, jasplakinolide, promoted neurite outgrowth in a CSPG-covered area and increased the ratio of neurites that broke through the CSPG boundary. These results provide further support that actin filament dynamics play a causal role in CSPG-mediated neurite repulsion and retraction during motility. Irreversible cell apoptosis occurred in the jasplakinolide-treated cells after 48 h of incubation, and this effect might be related to the jasplakinolide-mediated apoptosis pathway (Matsuki-Fukushima et al., 2012; Aida et al., 2016). However, this phenomenon does not negate the novel therapeutic proposal that neurite passage through a CSPG-containing chemical barrier can be promoted by increasing actin filament polymerization and/or decreasing actin depolymerization in the growth cone.

5. Conclusions

Collectively, the results of this study reveal that different spatial distributions of CSPGs induce different neurite regeneration behaviors in terms of neurite steering, branching and neurite retraction (Fig. 7). In addition, the reactions of neurites growing against CSPGs were mediated by the repulsive redistribution of actin filaments in growth cones, which allowed the neurites to avoid contact with the CSPGs. A linear distribution of CSPGs provides a route through which actin filament-associated filopodia or lamellipodia can extend and thereby influences neurite steering. Conversely, a dispersive distribution of CSPGs provides no space in which actin filament-associated structures can turn.

![Fig. 7](image-url) The effect of CSPGs on neurite growth is influenced by the spatial distribution of CSPGs. This diagram illustrates three different CSPG spatial distribution patterns and their specific effects on neurite outgrowth behavior. A. Under normal circumstances, DRG neuron growth will exhibit an extended neurite trunk with random axonal branching at the proximal and distal segments. B. As neurites gradually approach the edge of the CSPG boundary, the negative effects of CSPGs on guidance reject the ingrowth of the neurite, leading to neurite turning. C. When CSPGs cover the entire distal segment of the neurite trunk, the neurite-inhibiting effect of CSPGs inhibits neurite growth in addition to axonal branching from the distal segment. The neurite reacts with the structural collapse of the distal segment, while axonal branching from the proximal part of the neurite trunk is not inhibited. D. When CSPGs cover both the DRG soma and the proximal segment of the neurite trunk, only the axonal branching from the proximal part of the neurite is inhibited, while the continuous growth of the neurite trunk and axonal branching from the distal part of the neurite is not inhibited.
resulting in neurite stagnation or retraction and inhibited neurite sprouting. These results increase our understanding of these mechanisms and will promote the design of better nerve repair materials that can increase the orderliness of nerve regeneration. In combination with our observations regarding the regrowth of neurites on CSPG surfaces can increase the orderliness of nerve regeneration. In combination with our observations regarding the regrowth of neurites on CSPG surfaces after treatment with jaspilkinolide, our data suggest a potential therapeutical strategy in which actin stabilization and/or polymerization could be induced to promote the growth of nerve fibers that can pass through CSPG-enriched zones.

Compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (approval no. [2016]152).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by grants from the Chinese National Natural Science Foundation (No.31670986), the Foundation of Guangdong Province (No.2017A04050501017; 2014B020227001), National Key Research and Development Program of China (No.2016YFC1101603), the National Key Basic Research Program of China (No.2014CB542200), Science and Technology Planning Project of Guangdong Province, China (No.2015B090903060) and China Postdoctoral Science Foundation (No.2014T11101), International S&T Cooperation projects of Guangzhou, China (No.201807010082).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2018.05.023.

References

Aida, N., Ushikubo, T., Kobayashi, F., et al., 2016. Actin stabilization induces apoptosis in cultured porcine epithelial cell rest of Malasez. Int. Estod. J. 47 (9), 663-669.
Amano, M., Nakayama, M., Kaibuchi, K., 2010. Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. Cytoskeleton (Hoboken) 67 (9), 545-554.
Avram, S., Shaposhnikov, S., Buta, C., Menezas, M., 2014. Chondroitin sulfate proteoglycans: structure-function relationship with implication in neural development and brain disorders. Biomol. Res. Int. (2014), 642798.
Beller, J., Kulengowski, B., Koberai, E., et al., 2013. Comparison of sensory neuron growth cone and filopodal responses to structurally diverse aggrecan variants, in vitro. Exp. Neurol. 247, 143-157.
Challacome, J.F., Snow, D.M., LeTourneau, P.C., 1996. Actin filament bundles are required for microtubule reorientation during growth cone turning to avoid an inhibitory guidance cue. J. Cell Sci. 109 (P8), 2031-2040.
Dickendesher, T.L., Baldwin, K.T., Mironova, Y.A., et al., 2012. NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. Nat. Neurosci. 15 (5), 703-712.
English, A., 2005. Enhancing axon regeneration in peripheral nerves also increases functionally inappropriate reinnervation of targets. J. Comp. Neurol. 490 (4), 427-441.
Gause, T.M., Sivak, W.N., Marra, K.G., 2014. The role of chondroitinase as an adjuvant to peripheral nerve repair. Cells Tissues Organs 200 (1), 59-68.
Graham, J.B., Muir, D., 2016. Chondroitinase C selectively degrades chondroitin sulfate glycosaminoglycans that inhibit axonal growth within the endoneurium of peripheral nerve. PLoS One 11 (12), e0167682.
Graham, J.B., Neubauer, D., Xue, Q.S., Muir, D., 2007. Chondroitinase applied to peripheral nerve repair averts retrograde axonal regeneration. Exp. Neurol. 203 (1), 185-195.
Hagg, T., 2006. Collateral sprouting as a target for improved function after spinal cord injury. J. Neurotrauma 23 (4-3), 281-294.
Hahn, K., Sirdofsky, M., Brown, A., et al., 2006. Collateral sprouting of human epidermal nerve fibers following intracutaneous anatomy. J. Peripher. Nerv. Syst. 11 (2), 142-147.
Lang, B.T., Gregg, J.M., DePaul, M.A., et al., 2015. Modulation of the proteoglycan receptor PTPr promotes recovery after spinal cord injury. Nature 518, 404-408.
Lin, C.H., Espeulaco, E.M., Mooseker, M.S., et al., 1997. Myosin drives retrograde F-actin flow in neuronal growth cones. Neuron 16 (4), 769-782.
López-Alvarez, V.M.M., Modol, L., Navarro, X., Cobiánchi, S., 2015. Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury. Pain 156 (9), 1812-1825.
Man, A., Leach, K., Bannerman, P., 2014. Redirection of neurite outgrowth by coupling chondroitin sulfate proteoglycans to polymer membranes. Ann. Biomed. Eng. 42 (6), 271-281.
Massey, J.M., Hubscher, C.H., Wagoner, M.R., et al., 2006. Chondroitinase ABC digestion of the perineuronal net promotes functional collateral sprouting in the cuneate nucleus after cervical spinal cord injury. J. Neurosci. 26 (16), 4406-4414.
Masuda, T., Fukamachi, F., Takeda, Y., et al., 2004. Developmental regulation of notch- and growth cone motility and guidance. Mol. Cell. Neurosci. 23 (2), 217-227.
Matsuki-Fukushima, M., Hashimoto, S., Murakami, M., et al., 2012. The actin-specific reagent jaspilkinolide induces apoptosis in primary rat parietal cortex cells. Arch. Biol. 57 (5), 567-576.
McKeon, R.J., Juryniec, M.J., Buck, C.R., 1999. The chondroitin sulfate proteoglycans neurocan and phosphacan are expressed by reactive astrocytes in the chronic CNS glial scar. J. Neurosci. 19 (24), 10778-10786.
Medeiros, N.A., Burnett, D.T., Forscher, P., 2006. Myosin II functions in actin-bundle turnover in neuronal growth cones. Nat. Cell Biol. 8 (3), 215-226.
Monnier, P.F., Sierra, A., Schwab, J.M., et al., 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol. Cell. Neurosci. 22 (3), 319-330.
Muir, D., 2010. The potentiation of peripheral nerve sheaths in regeneration and repair. Exp. Neurol. 223 (1), 102-111.
Omontade, O., Pollitt, S., Zheng, J., 2017. Actin-based growth cone motility and guidance. Mol. Cell. Neurosci. 84, 4-10.
Pizorzoruo, T., Medini, P., Berardi, N., et al., 2002. Reactivation of ocular dominance plasticity in the adult visual cortex. Science 298 (5596), 1248-1251.
Ro, L.S., Chen, S.T., Tang, L.M., Jacobs, J.M., 1999. Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. Pain 79 (2-3), 265–274.
de Ruiter, G.C., Malesy, M.J., Aald, A.O., et al., 2008. Misdirection of regenerating motor axons after nerve injury and repair in the rat sciatic nerve model. Exp. Neurol. 211 (2), 339-350.
Schwegler, G., Schwab, M.E., Kaphammer, J.P., 1995. Increased collateral sprouting of primary afferents in the myelin-free spinal cord. J. Neurosci. 15 (4), 2756-2767.
Shen, Y., Tenasey, A.P., Busch, S.A., et al., 2009. PTPr is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. Science 326 (5952), 592-596.
Silver, J., Miller, J.H., 2004. Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5 (2), 146-156.
Smirnov, M.S., Cabral, K.A., Geller, H.M., et al., 2014. The effects of confinement on neuronal growth cone morphology and velocity. Biomaterials 35 (25), 6750-6757.
Snow, D.M., Lemmon, V., Carrino, D.A., et al., 1990. Sulfated proteoglycans in astroglial barriers inhibit neurite growth in vitro. Exp. Neurol. 109 (1), 111-130.
Snow, D.M., Mullins, N., Hynds, D.L., 2001. Nervous system-derived chondroitin sulfate proteoglycans regulate growth cone morphology and inhibit neurite outgrowth: a light, epifluorescence, and electron microscopy study. Micros. Resch. Tech. 54 (5), 273-286.
Williams, R.S., Cheng, L., Mudge, A.W., et al., 2002. A common mechanism of action for three mood-stabilizing drugs. Nature 417 (6886), 292-295.
Xu, H., Wu, F., Zhang, H., et al., 2017. Actin cytoskeleton mediates BMP2-Smad signaling via Talin in preeclampsia under simulated microgravity. Biochimie 138, 184-193.
Yu, P., Santiago, L.Y., Katagiri, Y., Geller, H.M., 2012. Myosin II activity regulates neurite outgrowth and guidance in response to chondroitin sulfate proteoglycans. J. Neurochem. 120 (6), 1117-1126.
Zhang, X.F., Schaefer, A.W., Burnett, D.T., et al., 2003. Rho-dependent contractile responses in the neuronal growth cone are independent of classical peripheral retrograde actin flow. Neuro 40 (3), 931-944.