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Research Paper

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

Jian-Long Zou[a], Jia-Hui Sun[c], Shuai Qiu[a], Shi-Hao Chen[d], Fu-Lin He[a], Jia-Chun Li[e], Hai-Quan Mao[f], Xiao-Lin Liu[a,b], Da-Ping Quan[d], Yuan-Shan Zeng[c], Qing-Tang Zhu[a,b,*]

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

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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).

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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-D-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-D-lysine coated 24 well dishes at a density of 1.0×10^5 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-D-lysine + vehicle strip) or CSPG pre-coated (poly-D-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% CO_2 and 92% humidity. The medium was changed every other day. All steps were performed are 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.

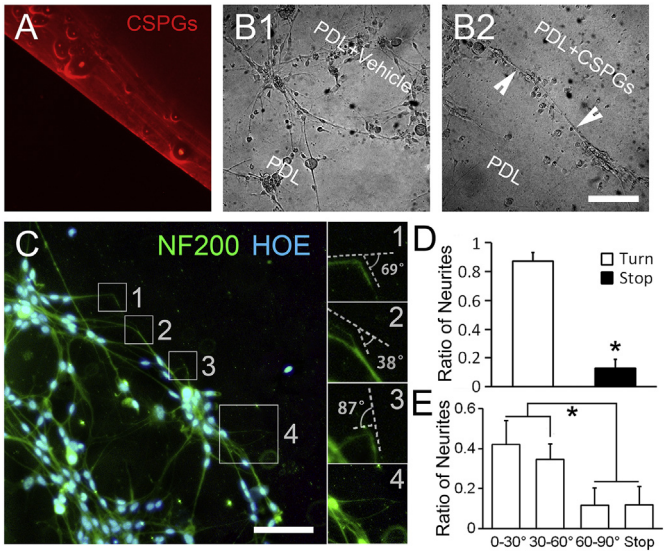

Fig. 1. The CSPG boundary rejects the ingrowth of neurites and leads to neurite turning. A In our cellulose strip-coating method, the CSPG boundary was created and retained on the bottom of culture dishes. B1 Vehicle strip-coating did not affect neurite outgrowth. The resulting neurites are shown spread and interwoven with each other in the field of view. B2 CSPG strip-coating inhibited neurite outgrowth on the CSPG-coated area. Some neurites grow essentially along the boundaries of the CSPGs on the PDL side. C Immunofluorescence staining for NF200 showed that neurites turned at the CSPG boundary area. The enlarged region of interest (ROI) shows neurites and their turning angles (numbers 1, 2, and 3) and neurites without any obvious turning (number 4). D A histogram shows that the ratio of angular neurites was significantly higher than the ratio of neurites that did not turn. E A histogram of the turning angle distribution showing that a larger proportion of neurites showed a small steering angle. *$p < 0.01$. Scale Bar = 100 μm in A–B2, 50 μm in C.

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-D-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-D-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.