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The roles of neuronal and glial precursors in overcoming chondroitin sulfate proteoglycan inhibition

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Abstract

The extension of axons through the major inhibitory component of the glial scar, chondroitin sulfate proteoglycans (CSPG), remains a key obstacle for regeneration following spinal cord injury (SCI). We have previously shown that transplants composed of neuronal and glial restricted precursors (NRP and GRP respectively) promote regeneration and connectivity in the injured spinal cord (Bonner et al., 2010; 2011), however, little is known about the properties of these precursors at a cellular level. We now report that NRP-derived neurons, in contrast to dorsal root ganglion (DRG) neurons, have the ability to extend axons and cross over from a permissive substratum (laminin) onto inhibitory CSPG *in vitro*. Growth cones of neurons derived from NRP, compared to DRG, exhibit significantly lower levels of the CSPG receptors protein tyrosine phosphatase sigma (PTP σ) and leukocyte common antigen-related phosphatase (LAR). GRP-conditioned medium prepared from the same cell densities did not affect the response of primary sensory neurons to CSPG confirming that the ability of NRP-derived neurons to cross onto CSPG is determined intrinsically. However, GRP-conditioned medium collected from high density cultures increased the probability of DRG axons to cross from LN onto CSPG and increased the length of DRG axons extending on CSPG. Collectively, these results suggest that (1) neurons derived from NRPs are intrinsically insensitive to CSPGs due to low levels of receptor expression, and (2) high levels of factors secreted by GRP can reduce the inhibitory effects of CSPG and promote axonal growth. These observations provide mechanistic insights into the specific roles of NRPs and GRPs in promoting regeneration and repair following SCI.

Keywords

Neuronal restricted precursors; glial restricted precursors; spinal cord injury; receptor PTP σ ; LAR; axon outgrowth

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Introduction

The limited regenerative capacity of CNS neurons and the formation of an inhibitory environment at the injury site represent major challenges for regeneration and repair of spinal cord injury (SCI) (Silver and Miller, 2004; Fitch and Silver, 2008). SCI induces the formation of a glial scar composed of several growth-inhibitory molecules, including chondroitin sulfate proteoglycans (CSPGs) (Sandvig et al., 2004; Fawcett, 2006; Rhodes and Fawcett, 2004). CSPGs are upregulated at the lesion site of CNS injuries and present a barrier for axon regeneration (Jones et al., 2003; Fitch and Silver, 2008; Properzi et al., 2003, 2005). *In vitro* model systems designed to mimic the distribution of CSPG at the lesion site have demonstrated that when axons are initially growing on a permissive substratum they stop/turn when they encounter CSPGs (Hynds and Snow, 1999, 2001; Dou and Levine, 1994; Schmalfeldt et al., 2000; Yu and Bellamkonda, 2001; Tom et al., 2004).

The use of embryonic neurons for cell transplantation is a promising therapeutic strategy for SCI with the potential of repairing damaged circuitry by providing replacement neurons and promoting connectivity. Previous studies have been successful in generating a neuronal relay across the injured spinal cord using a combination of neuronal (NRP) and glial restricted precursor (GRP) cell transplants and neurotrophin support (Bonner et al., 2010; Bonner et al., 2011). In these studies, the axons of sensory neurons extended into the injury site and formed synaptic connections with graft-derived neurons. In addition, axons of graft-derived neurons were guided out of the injury site and into the dorsal column nucleus to form a functional relay. Further elucidation of the respective roles of NRP and GRP in reconnecting the injured spinal cord will yield insights into how these cells may be used in a therapeutic context.

We have previously shown that NRP-derived neurons will extend axons on uniform CSPG-coated substrata (See et al., 2010). The goal of the present study was to determine if the axons extending from these neurons could cross over from a permissive substratum into a territory enriched with CSPGs. We report that the axons of NRP-derived neurons readily cross into CSPG enriched substrata *in vitro*. The ability of these axons to cross onto CSPG correlates with greatly decreased levels of CSPG receptors in the growth cones of these neurons, relative to primary sensory neurons that do not cross onto CSPG. Furthermore, conditioned medium from GRP cultures, the same density as in co-cultures of NRP/GRP, does not affect the crossing of DRG axons onto CSPG. However GRP-conditioned medium from high density cell cultures reduced the inhibitory effects of CSPG on DRG neurons. These results indicate (1) NRP-derived neurons are intrinsically insensitive to CSPG and (2) GRPs secrete factors that promote axon extension of sensory neurons into inhibitory regions of CSPG. Collectively, these results identify properties of NRPs and GRPs that are beneficial for cell transplantation-based strategies designed to promote regeneration and connectivity in SCI.

Materials and Methods

NRP/GRP cultures for CSPG border crossing assays

Cultures of NRP and GRP were obtained from spinal cords of embryonic day 13.5 rat from three separate litters following published protocols (Lepore et al., 2004). Mixed cells of NRP/GRP were plated at a concentration of 10,000 cells per coverslip (18×18mm) containing prepared CSPG borders. Control basal medium contained DMEM-F12 (Gibco), B27 supplement (20μL/mL; Gibco), Pen-Strep (100 IU/mL; Gibco), BSA (1mg/mL; Sigma), N2 supplement (10μl/mL; Gibco), bFGF (10μg/mL). Control basal medium was supplemented with NT-3 (10μg/mL; Peprotech) and retinoic acid (10μM; Sigma) to

promote neuronal differentiation. Cultures were fixed 24 hours after plating for analysis. Substratum preparation is described below.

DRG cultures

Embryonic day 7 chicken dorsal root ganglia were cultured as explants and supplemented with 20ng/mL nerve growth factor (R&D Systems, Minneapolis, MN) to promote neuronal outgrowth following published protocols (Lelkes et al., 2006). Dissociated neurons from dorsal root ganglia were obtained from rat embryonic day 19 (E19) and supplemented with 50ng/mL nerve growth factor. Both sets of neurons (chick and rat) were cultured either in (1) control basal medium or (2) conditioned medium (described below).

NRP/GRP- and GRP-conditioned medium

To address if cells in mixed NRP/GRP cultures are secreting factors that influence NRP-derived axons in crossing onto CSPG, conditioned medium was collected from both mix cultures of NRP/GRP and isolated cultures of GRP. In order to assure the cell densities (10,000 cells plated on 18×18mm coverslips in 500μl of medium) in cultures used to generate conditioned medium were equivalent to the density of NRP/GRP cultures used in the NRP crossing border assays, NRP/GRP- and GRP-conditioned medium was collected 24 hours after culturing NRP/GRP or GRP at the same density (see above) and on the same substrata (see below) as prepared in the crossing assays. The conditioned medium was then transferred to rat and chick DRG cultures for 24 hours.

GRP high density conditioned medium

To determine if GRPs secrete factors that alter the response of primary DRG axons to CSPG borders, GRP-conditioned medium was also tested from high density cultures (GRP-HDCM). Purified rat GRP (P5) were plated onto T25 flasks coated with PL + LN and allowed to recover overnight in basal medium supplemented with bFGF (40 ng/mL; Peprotech). The following day the cells were washed with HBSS and cultured for 3 days in basal medium supplemented with bFGF (30ng/mL). On the third day media was harvested and utilized for further experimentation. Final concentration of cells was approximately 1 million GRP per mL.

To further investigate the properties of GRP-secreted factors, dilution series were performed with GRP-HDCM. DRG rat neurons were cultured on prepared CSPG borders and cultured with varying dilutions of GRP-HDCM; 100% GRP-HDCM, 50% GRP-HDCM, 10% GRP-HDCM, or 100% control basal medium supplemented with bFGF (30ng/mL). The concentrations of GRP-HDCM were acquired by diluting GRP-HDCM with control medium.

CSPG Border Preparation

Coverslips (18×18mm) were coated with 100μg/mL of poly-D-lysine (PL) (70 KD MW; Sigma) overnight. The following day, coverslips were washed and dried. Using fine forceps, a 1mm thick strip of filter paper (Fisherbrand P5) saturated with 3μL of 100μg/ml Chondroitin Sulfate Proteoglycan (combination of brevican, phosphacan, aggrecan, and versican, CC117; Millipore) was placed on the coverslip. The filter paper was removed once it had completely dried. Following washing, 15μg/mL laminin (LN) (Invitrogen) was applied to the coverslip for 1 hour at 37°C. Increasing the LN/CSPG ratio directly affects growth cone advance onto the inhibitory CSPG (Snow and Letourneau, 1992), therefore we tested various concentrations of CSPG (10–200μg/mL) with DRG neurons to determine the appropriate concentration for turning response to CSPG border. For analysis of border

assays, we used a concentration of CSPG (100 μ g/mL) which showed turning behavior of DRG axons greater than 95%.

Pre-treatment of CSPG borders with conditioned medium

Coverslips containing prepared CSPG borders were pre-treated with 500 μ l of either (1) GRP-HDCM or (2) control basal medium for 24 hours. Coverslips were washed three times with control basal medium and used for plating rat E19 DRG. Following pre-treatment of substratum, dissociated sensory neurons were cultured with control basal medium supplemented with 50ng/mL NGF. For positive control, DRG neurons were cultured with GRP-HDCM in parallel.

Immunocytochemistry

Cultures were fixed with 4% PFA (Electron Microscopy Sciences) containing 5% sucrose for 15 min, and incubated in blocking solution containing 10% goat or donkey serum (Gibco) and 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) for 20 min. To visualize the CSPG border, coverslips were stained using mouse (IgM) anti-CSPG (clone CS-56, 1:500; Sigma). To label neurons, coverslips were stained with either (1) the neuronal specific primary antibody TUJ1 (rabbit monoclonal 1:1000; Covance) for NRP/GRP and rat DRG cultures, or (2) fluorescein-conjugated anti-tubulin monoclonal antibody (DM1A clone, 1:100; Sigma) for chick DRG cultures. For receptor staining, cultures were incubated with anti-beta III tubulin (TUJ1) and either anti-hPTPR σ (goat IgG 1:100; AF3430, R&D Systems) or anti-LAR (H-70) (rabbit IgG 1:200; sc-25434, Santa Cruz). All cultures were stained with primary antibodies overnight at 4°C, followed by washing and staining with secondary antibodies for 1hr at room temperature. All cultures were stained for DAPI (Vectashield with Dapi) to visualize nuclei of all cells.

Imaging and Analysis

For consistency of analysis between cell cultures, images were taken at regions identifying the border between LN and LN + CSPGs (using CS-56 antibody) in which cell density was sparse in order for the number of individual axons and cell bodies to be accurately calculated. All images were obtained and analyzed using an Axiovert 200M microscope (Carl Zeiss) equipped with an Orca ER camera (Hamamatsu). For this study an axon is defined as >25 μ m in length. Axon crossing behavior was quantified by counting the number of individually identified axons (TUJ1 positive) either (1) crossing or (2) turning after contact with the CSPG border. We analyzed only axons that contained cell bodies positioned at least 20 μ m (equivalent to two cell bodies) from the border. Axon fasciculation has been shown to assist axon crossing onto CSPGs (Snow et al., 2003) therefore we only analyzed individually identified axons. For DRG analysis, experiments were performed in triplets and independently repeated at least three times. For NRP/GRP analysis, experiments were performed using 5 different samples and independently repeated at least four times. Statistical analysis was performed using X^2 test, measuring raw categorical data for crossing behavior and direction of axon outgrowth.

To determine if CSPG affects the length of axons extending from NRP/GRP cultures, images were acquired of TUJ1 positive axons positioned on CSPG (indicated by CS-56 staining) or off CSPG (indicated by absence of CS-56 staining) using double stacks. The length of the axon was measured in μ m using the interactive measurement module of Zeiss AxioVision Software. The axon was defined as the longest process extending from the cell body with a distance greater than 10 μ m. Statistical analysis was performed with a Mann Whitney test using GraphPad InStat.

To quantify staining intensity of CSPG receptors (PTP σ and LAR), images were acquired using triple channel stacks in identical register. Measurements of PTP σ /LAR total staining intensity were performed at the growth cone, defined as the structure comprised by lamellipodia and filopodia encompassing the distal most 15 μ m of the axon. The measurements of staining intensity were determined using the interactive measurement module of the AxioVision Software. Total staining intensity was determined by integrating the pixel intensities within the area of measurement. Background intensity values were subtracted from all images. Statistical analysis for both receptors was performed with a Mann-Whitney test using GraphPad InStat.

Results

NRP-derived neurons cross borders between LN and CSPGs

In order to determine the response of the axons of NRP-derived neurons initially extending on a permissive substratum with CSPGs, we used a border assay composed of a permissive LN-coated side and a non-permissive LN + CSPG-coated side. When plated on the LN-coated side of the border, growth cones of various neuronal cell types stop or turn at the CSPG border to remain on the LN (Snow et al., 2001; Dou and Levine, 1994; Hynds and Snow, 1999, 2001; Schmalfeldt et al., 2000; Yu and Bellamkonda, 2001). The CSPGs used in our experiments were composed of brevican, phosphacan, aggrecan, and versican, which all have been shown to be highly upregulated in the lesion site following CNS injury (Jones et al., 2003; Afshari et al., 2010). The percentage of axons crossing was quantified by counting the number of TUJ1 positive axons either turning or crossing after contact with CSPGs. Only axons isolated from surrounding cells were scored in order to eliminate any influence of cell-to-cell contact. As expected, the axons extending from chick embryonic day 7 and rat embryonic day 19 DRGs on LN did not cross onto the CSPG-coated region (100 μ g/mL), but rather turned and remained on the LN (Figure 1A). In contrast, axons extending from NRP-derived neurons, in a mixed culture containing GRP, crossed the border and extended onto the CSPGs (Figure 1B). We found that 78% of axons extending from NRP-derived neurons crossed the border and extended onto CSPGs (Figure 1C). In contrast, 4% (chick) and 4% (rat) of axons extending from sensory neurons crossed the border and extended onto CSPGs (Figure 1C; X^2 $p < 0.0001$ for NRP compared to both chick and rat DRG, statistical analysis performed on raw categorical data).

Analysis of turning behavior was performed on fixed cultures, therefore it is possible that axons in the NRP/GRP cultures retract after contact with CSPG and redirect their extension away from the border. As a result the crossing analysis would score only axons that did not retract following contact with CSPGs. In order to rule out bias in the method for sampling the interactions between the axons of NRP and the CSPG borders, we divided the neuronal cell bodies into 4 equal quadrants and calculated the direction in which axons extended in relation to the CSPG border (Figure 2A). We found that there was no directional preference of axon extension from the NRP cell body and the position of the border (Figure 2B; $p = 0.6$ for all comparisons, X^2 test performed on raw categorical data). Thus, the NRP-derived neurons scored for border crossing do not represent a biased subset of NRP axons.

CSPGs do not affect the length of NRP-derived axons

The length of axons derived from NRP neurons is not adversely affected by substrata uniformly coated with 5 μ g/mL CSPG (See et al., 2010). Those experiments were performed using nitrocellulose and polylysine as the background substratum coating. In our experiments, we coated coverslips with LN to promote survival and outgrowth of neurons. Increasing the LN/CSPG ratio affects growth cone advance onto the inhibitory CSPG (Snow and Letourneau, 1992), therefore we required a higher concentration of CSPG (100 μ g/mL)

to establish an avoidance response by sensory neurons to the CSPG. To determine if the concentration of CSPGs used in our experiments could affect the length of the axons of NRP-derived neurons, we measured the length of axons extending from NRP-derived neurons positioned on both sides of the border (LN versus CSPG) that did not however interact with the border (Figure 2C). The mean length of axons extending from NRP-derived neurons was not significantly different between neurons on LN ($158\mu\text{m} \pm 11 \text{ SEM}$) compared to neurons on LN + CSPG ($148\mu\text{m} \pm 12 \text{ SEM}$) (Figure 2D; $p = 0.3946$, Mann Whitney Test). The data indicate that consistent with our previous study (See et al., 2010), using different substrata, the extension of NRP axons is not adversely affected by growing on uniform CSPG substrata.

The growth cones of NRP-derived neurons exhibit lower levels of the CSPG receptors PTP σ and LAR than primary sensory neurons

Given the significant difference in response to CSPGs by NRP and DRG neurons, we next sought to analyze possible NRP-intrinsic mechanisms that may be responsible for the insensitivity of NRP-derived neurons to CSPGs. CSPGs elicit cellular responses through membrane bound receptor systems, which represent the first step in the transduction of the CSPG signal. The receptor PTP σ has been identified as a receptor for inhibitory CSPGs (Shen et al., 2009). Both *in vitro* and *in vivo* studies have shown disruption of the PTP σ gene to enhance axon outgrowth through regions enriched in CSPG (Shen et al., 2009; Coles et al., 2011). Based on the insensitivity of NRP-derived neurons towards CSPGs, we investigated the levels of CSPG receptors in the distal axons and growth cones of NRP-derived neurons through quantitative immunocytochemistry using an antibody against PTP σ (Coles et al., 2011). As expected, both rat and chick sensory neurons expressed the receptor PTP σ (Figure 3A). Although at clearly decreased levels relative to the primary neurons, NRP-derived neurons also expressed the PTP σ receptor (Figure 3A). The receptor was observed in the cell body, and throughout the axon and growth cone of all three types of neurons (data not shown). PTP σ was detected in both, growth cone filopodia and lamellipodia, the cellular structures that mediate growth cone guidance in response to CSPGs (Gomez and Letourneau, 1994; Challacombe et al., 1997). Measurement of the total PTP σ content of growth cones revealed that the growth cones of NRP-derived neurons exhibit 74% less PTP σ compared to growth cones of E19 rat DRG neurons (integrated pixel intensities value, $5447 \pm 902 \text{ SEM}$ and $21210 \pm 3312 \text{ SEM}$, respectively) (Figure 3B; $p < 0.0001$, Mann-Whitney Test).

LAR is an additional member of the protein receptor phosphatase family that has been shown to play an important role in the regulation of axon extension across inhibitory CSPGs (Fisher et al., 2011). Increased neurite outgrowth over CSPGs has been shown using both selective LAR blocking peptides and LAR knock-out mice (Fisher et al., 2011). Immunocytochemistry revealed staining of LAR in all three types of neurons (rat DRG, chick DRG, NRP; Figure 4A). Similar to PTP σ , LAR was detected in the cell bodies, along axons and at the growth cones. In growth cones, the LAR receptor was also detected in filopodia and lamellipodia. Quantitative immunocytochemistry, as performed for PTP σ , revealed that the growth cones of NRP-derived neurons exhibit a 95% reduction in staining levels for LAR content compared to growth cones of E19 rat DRG neurons (integrated pixel intensities value, $1640 \pm 314 \text{ SEM}$ and $34153 \pm 6599 \text{ SEM}$, respectively) (Figure 4B; $p < 0.0001$, Mann-Whitney Test). Thus, relative to primary neurons, NRP growth cones exhibit greatly decreased levels of CSPG receptors.

The ability of NRP-derived axons to cross onto CSPG in mixed NRP/GRP cultures is independent of contact with GRPs and secreted factors

The presence of GRPs in the mixed NRP/GRP culturing experiments described in Figure 1 may contribute to the response of NRP axons to CSPG borders through either contact mediated mechanisms or the release of soluble factors into the medium. To eliminate any contact mediated effects of GRP, for the data shown in Figure 1, we only sampled isolated NRP-derived axons and neurons, not in contact with any other cells. Thus, it is not likely that GRP-NRP contact is responsible for the ability of NRP axons to cross onto CSPGs. To determine if the GRPs have an indirect non-cell contact mediated de-sensitizing effect on axons to CSPGs, rat and chick DRGs were cultured in conditioned medium from isolated pure GRP cultures, not containing NRPs, plated at the same cell densities as the NRP crossing assays (see Methods for preparation of conditioned medium). The GRP-conditioned medium did not affect the turning response of DRG axons at CSPG borders (Figure 5A, B; X^2 $p = 0.94$; $p = 0.16$ for rat and chick DRG respectively). Similarly, conditioned medium collected from cell-density matched mixed cultures of NRP/GRP did not affect the turning response of DRG axons to CSPG compared to control medium (Figure 5B; X^2 $p = 0.82$; $p = 0.44$ for rat and chick DRG respectively). These data indicate that the lack of turning of NRP axons at CSPG borders in mixed cultures of NRP/GRP cells (Figure 1) is not attributable to factors secreted by the cells into the medium.

GRP-conditioned medium from high density cultures (GRP-HDCM) reduces the inhibitory effects of CSPG by sensory axons

Although the data described above (Figure 5) rule out a role of secreted factors in the crossing of axons from NRP-derived neurons at CSPG borders, we sought to further test the hypothesis that GRPs may release factors that can promote axon crossing in neurons that are sensitive to CSPG inhibition. Rat embryonic DRGs were cultured with GRP-conditioned medium collected from high density populations (see Methods for details on the preparation of GRP-HDCM). DRG axons cultured with the GRP-HDCM were more likely to cross the inhibitory CSPG barrier compared to axons cultured with control medium (Figure 6A, B). When presented with a border consisting of the interface between LN and LN + CSPG, 16% of axons extending from DRG neurons cultured with GRP-HDCM crossed the border and extended onto CSPGs compared to 4% of axons cultured with control medium (Figure 6B, X^2 test $p < 0.0001$). Furthermore, the effect of GRP-HDCM in promoting axon outgrowth onto CSPGs was dose dependent. The percentage of axons to cross from a permissive substratum onto inhibitory CSPG significantly decreased with increasing dilutions of GRP-HDCM. The percentage of DRG axons crossing onto CSPGs significantly decreased from 16% with GRP-HDCM to 8% with GRP-HDCM diluted by half with control medium (X^2 test comparing 50% GRP-HDCM to 100% GRP-HDCM $p = 0.0006$; comparing 50% GRP-HDCM to control $p = 0.037$). There was no significant difference in the number of axons crossing onto CSPG between neurons cultured with 100% control medium compared to 90% control medium and 10% GRP-HDCM (4% and 3% respectively; X^2 test $p = 0.8$).

In addition to the enhanced crossing of DRG axons, we also analyzed the effects of GRP-HDCM on the percentage of DRG neurons that extend axons and the length of the axons on uniform CSPG substrata. Similar to the crossing behavior, GRP-HDCM had a dose dependent effect on both the percentage of neurons to extend axons on CSPG and the length of those axons. We found that 76% of DRG neurons cultured with GRP-HDCM extended axons on CSPG compared to 39% of neurons cultured with control medium (Figure 6C, D; X^2 test $p < 0.0001$). The percentage of neurons that extended axons on CSPG directly correlated with the concentration of GRP-HDCM (Figure 6D). 68% of neurons cultured with 50% GRP-HDCM extended axons on CSPG and 55% of neurons cultured with a dilution containing 10% GRP-HDCM extended axons on CSPG (Figure 6D; X^2 test compared to

control, 10% GRP-HDCM $p = 0.0015$; 50% GRP-HDCM $p < 0.0001$). The GRP-HDCM also had a dose dependent affect on the length of axons extending from DRG neurons plated on CSPG (Figure 6E, F). The length of axons cultured with 100% GRP-HDCM averaged $75\mu\text{m} \pm 3 \text{ SEM}$, compared to $57\mu\text{m} \pm 3$ cultured with a dilution of 50% GRP-HDCM, $44\mu\text{m} \pm 3$ cultured with a dilution containing 10% GRP-HDCM and $28\mu\text{m} \pm 3$ cultured with control medium (Figure 6F; Mann-Whitney Test, all conditions compared to control, 10% GRP-HDCM $p = 0.0007$; 50% and 100% GRP-HDCM $p < 0.0001$).

Pre-treatment of CSPG substrata with GRP-HDCM lessens the inhibitory effects of CSPGs on sensory axons

The experiments performed with GRP-conditioned medium suggest that GRPs secrete factors that decrease the response of sensory axons to CSPGs. To determine if secreted factors released by GRPs are directly affecting the sensory neurons or modifying the inhibitory properties of the CSPG substratum, we performed conditioned medium wash-out experiments. If GRP-secreted factors in the conditioned medium are altering the inhibitory properties of the CSPG substratum, then pretreatment of CSPG substrata followed by washout of the conditioned medium prior to culturing of the neurons should be as effective as culturing the neurons and substrata in the continued presence of conditioned medium. Coverslips with prepared CSPG borders were treated for 24 hours with either GRP-HDCM or control medium. The conditioned or control medium was then removed, washed, and the substrata were subsequently used for culturing with embryonic rat DRG neurons in control medium. The percentage of DRG axons that crossed onto CSPG was significantly increased by pre-treatment of CSPG substrata with GRP-HDCM (23%) compared to pre-treatment of substrata with control medium (3%) (Figure 7A, B; X^2 test $p < 0.0001$). The increase in axon crossing with pre-treatment of substratum with GRP-HDCM (23%) is comparable to the increase in axon crossing when cultured with GRP-HDCM (18%). Additionally, the percentage of neurons that extended axons on CSPG was significantly increased when cultured on substratum pre-treated with GRP-HDCM (90%) compared to control medium (45%) (Figure 7C, D; X^2 test $p < 0.0001$). Pre-treatment with GRP-HDCM also had an effect on the length of axons extending on CSPG. The average length of axons on CSPG increased from an average of $58\mu\text{m} \pm 5$ with pre-treatment of substrata with control medium to an average of $132\mu\text{m} \pm 8$ with pre-treatment of substrata with GRP-HDCM (Figure 7E, F; Mann-Whitney Test $p < 0.0001$). There was no significant difference found between the percentage of axons that extended axons on CSPG (X^2 test $p = 0.13$) and the length of axons on CSPG (T-Test $p = 0.64$) when comparing pre-treatment of substrata with GRP-HDCM and culturing of DRG neurons in the continuous presence of GRP-HDCM. Together the data indicate that GRPs secrete factors that affect the inhibitory properties of CSPG and are not acting directly on the sensory neurons.

Discussion

The accumulation of CSPG in the glial scar inhibits axon outgrowth and is partially responsible for failed neuronal regeneration following injury (Matsui and Oohira, 2004; Sandvig et al., 2004; Tom et al., 2004). In addition to the inhibitory environment surrounding the injury site, the limitations of intrinsic axon growth programs of injured adult CNS neurons negatively influence the regeneration process (Blackmore and Letourneau, 2006; Borisoff et al., 2000). In this study we identify NRPs as generating neurons that are intrinsically insensitive to CSPGs and capable of robust axon extension. We have previously reported that neurons derived from mixed cultures of NRP and GRP are less sensitive to the inhibitory proteoglycan environment both *in vivo* (Lepore and Fischer, 2005) and *in vitro* (See et al., 2010). The current study extends these previous investigations by determining that neurons derived from these cultures are capable of extending axons that cross over from

a permissive substratum into regions enriched with inhibitory CSPGs *in vitro*. The CSPGs used in this study represent a mixture of brevican, phosphacan, aggrecan, and versican, and have been previously used to examine crossing behavior of neurons *in vitro* (Hynds and Snow, 1999, 2001; Curinga et al., 2007). It will be of interest in future studies to determine the relative sensitivity of NRP axons to individual CSPG species.

When evaluating the crossing behavior of axons it is important to take into account the differences between cultures of sensory neurons and NRP-derived neurons. In NRP/GRP mixed cultures approximately 5% of the cells are NRP and extend axons. In contrast, a single DRG explant contains mostly neurons that provide hundreds of axons. However, the purpose of analyzing the response of sensory neurons to CSPG borders was not to directly compare them to NRP-derived neurons, but to set a control baseline for inhibitory responses to CSPG by responsive axons. Considering the average percentage of neurons crossing the CSPG border, 78% NRP-derived axons compared to 4% rat and chicken sensory axons, the results strongly indicate that NRP-derived neurons are insensitive to the inhibitory CSPG. In contrast, the axons of NRP respond to attractive cues, and can be guided by gradients of BDNF *in vivo* (Bonner et al., 2010; Bonner et al., 2011). Similarly, NRPs extend axons on laminin which requires integrin signaling. Thus, the axons of NRP are not generally unable to respond to extracellular signals, but rather are selectively insensitive to CSPG inhibition. It remains to be determined whether NRP axons will be affected by other signals that can inhibit regeneration (e.g., semaphorins).

In order to gain insights on the lack of a turning response by NRP-derived neurons at CSPG borders, we investigated whether these cells express the two phosphatase receptors, PTP σ and LAR, both of which have been shown to mediate the inhibitory effects of CSPGs (Shen et al., 2009; Coles et al., 2011; Fisher et al., 2011). We found that NRP-derived neurons, similar to primary sensory neurons, express both the PTP σ and LAR receptors. However, quantitative immunocytochemical analysis of the levels of PTP σ and LAR in the growth cones of NRP-derived neurons revealed 74% and 95% less staining relative to rat DRG neurons. This observation suggests that the lack of turning response by NRP-derived neurons at CSPG borders may be a result of low levels of the expression of the CSPG receptors, PTP σ and LAR. However, our study does not rule out additional possible differences between NRP and primary neurons in downstream signaling mechanisms.

Although this study identifies NRP-derived neurons as being largely insensitive to CSPGs, a full understanding of the mechanism by which NRP-derived neurons extend axons onto CSPGs will require further investigation. Multiple studies have reported insights into the mechanisms involved in the regulation of CSPG inhibition, such as myosin II (Yu et al., 2011) and EGFR (Koprivica et al., 2005), along with signaling pathways involving Akt (Fisher et al., 2011) and integrins (Tan et al., 2011). In future studies it will be important to elucidate additional intrinsic properties that may be unique to the insensitivity of NRP-derived neurons to CSPGs. Do NRP-derived neurons have increased amounts of integrin receptors or decreased levels of EGFR compared to other neurons? Do NRP-derived neurons use the molecular motor protein myosin II for guidance? In future studies we will aim to address the roles of additional receptor systems, signaling pathways and cytoskeletal effectors in the lack of a response to CSPGs by NRPs, and the ability of NRPs to respond to additional extracellular signals, both growth promoting and inhibitory.

Immature glia can reduce the inhibitory glial scar and promote neuronal survival and outgrowth (Hill et al., 2004; Davies et al., 2006). Since in our border crossing studies we only analyzed NRPs that were not in contact with GRPs, the results indicate that NRP-derived axons are able to cross CSPG barriers without neuron-GRP contact-dependent mediated facilitation. Furthermore, our results show that both NRP/GRP- and GRP-

conditioned medium, at similar cell densities as the NRP crossing assays, does not contain sufficient levels of secreted factors that promote the extension of primary sensory axons into CSPG enriched inhibitory regions.

However, our results also show that DRG axons cultured with GRP-HDCM are more likely to extend axons from a permissive substratum onto an inhibitory CSPG-enriched territory, and that GRP-HDCM increases both the percentage of sensory neurons that extend axons and the length of axons on CSPGs. The results of experiments using GRP-HDCM to treat CSPG substrata prior to culturing of the neurons on the substrata, in the absence of further GRP-HDCM exposure, indicate that GRP secreted factors promote sensory axon growth through a regulation of the inhibitory properties of CSPGs. These data also argue against GRP soluble factors acting directly on the sensory neurons and affecting their response to CSPGs. There are multiple possible mechanisms by which factors secreted by GRPs may be altering the inhibitory properties of CSPGs. The secreted factors may be affecting the CSPG by masking or degrading the inhibitory components responsible for the avoidance response by axons, or contain substratum binding factors that counter the effects of CSPGs on axons. For example, CSPGs inhibit axon extension, while Heparan Sulfate Proteoglycans (HSPGs) promote axon extension (Su and Elam, 2003). HSPGs function as co-receptors for various growth factors, including FGF (Schlessinger et al., 1995), and compete with CSPGs for binding to PTP σ (Coles et al., 2011). Future investigations are aimed to determine if the secreted factors produced by the GRPs bind to the substratum and subsequently promote axon extension by overriding the CSPG pathway and activating the HSPG pathway. Secreted matrix metalloproteinases (MMPs) degrade extracellular matrix proteins. Another possibility relates to the action of MMP-9, which is produced in a variety of cell lines and alters several biochemical properties of CSPG (Windberg et al, 2000; Malla et al., 2008). Indeed, both MMP-9 and MMP-2 have been linked to enhancing axon outgrowth in degenerated nerves (Zuo et al., 1998; Ferguson and Muir, 2000). Future studies will also address whether the secreted factors produced by GRPs are part of the MMP family.

Conclusions

The data indicate that neurons derived from NRP/GRP mixed cultures are intrinsically capable of extending axons across an inhibitory CSPG environment *in vitro*. Our results suggest the insensitivity of NRP-derived neurons to CSPG may be due to the relatively low levels of PTP σ and LAR receptors at the growth cones of these neurons. Additionally, our results show that GRPs secrete factors that can promote axon outgrowth onto CSPG-enriched regions, and function by modifying or degrading the CSPGs. The intrinsic insensitivity of NRP-derived neurons to CSPGs, and the identification of CSPG modulatory factors secreted by GRPs, both contribute to the growing body of evidence that these cells may be ideally suited for use in promoting regeneration and repair of spinal cord injury.

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Abbreviations

NRP	neuronal restricted precursor
GRP	glial restricted precursor

DRG	dorsal root ganglia
NGF	nerve growth factor
bFGF	basic fibroblast growth factor
CNS	central nervous system
SCI	spinal cord injury
CSPG	chondroitin sulfated proteoglycans
LN	laminin
PL	poly-L-lysine
PTPσ	protein tyrosine phosphatase sigma
LAR	leukocyte common antigen-related phosphatase
HDCM	high density conditioned medium
SEM	standard error mean

References

- Afshari FT, Kwok JC, White L, Fawcett JW. Schwann cell migration is integrin-dependent and inhibited by astrocyte-produced aggrecan. *Glia*. 2010; 58:857–69. [PubMed: 20155822]
- Blackmore M, Letourneau PC. Changes within maturing neurons limit axonal regeneration in the developing spinal cord. *J Neurobiol*. 2006; 66:348–60. [PubMed: 16408302]
- Bonner JF, Blesch A, Neuhuber B, Fischer I. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. *Neurosci Res*. 2010; 88:1182–92.
- Bonner JF, Connors TM, Silverman WF, Kowalski DP, Lemay MA, Fischer I. Grafted Neural Progenitors Integrate and Restore Synaptic Connectivity across the Injured Spinal Cord. *J Neurosci*. 2011; 31:4675–86. [PubMed: 21430166]
- Borisoff JF, Pataky DM, McBride CB, Steeves JD. Raphe-spinal neurons display an age-dependent differential capacity for axon outgrowth compared to other brainstem-spinal populations. *Exp Neurol*. 2000; 166:16–28. [PubMed: 11031080]
- Challacombe JF, Snow DM, Letourneau PC. Dynamic microtubule ends are required for growth cone turning to avoid an inhibitory guidance cue. *J Neurosci*. 1997; 17:3085–95. [PubMed: 9096143]
- Coles CH, Shen Y, Tenney AP, Siebold C, Sutton GC, Lu W, Gallagher JT, Jones EY, Flanagan JG, Aricescu AR. Proteoglycan-specific molecular switch for RPTP σ clustering and neuronal extension. *Science*. 2011; 332:484–8. [PubMed: 21454754]
- Curinga GM, Snow DM, Mashburn C, Kohler K, Thobaben R, Caggiano AO, Smith GM. Mammalian-produced chondroitinase AC mitigates axon inhibition by chondroitin sulfate proteoglycans. *J Neurochem*. 2007; 102:275–288. [PubMed: 17394547]
- Davies JE, Huang C, Proschel C, Noble M, Mayer-Proschel M, Davies SJ. Astrocytes derived from glial-restricted precursors promote spinal cord repair. *J Biol*. 2006; 5:7. [PubMed: 16643674]
- Dou CL, Levine JM. Inhibition of axon growth by the NG2 chondroitin sulfate proteoglycan. *J Neurosci*. 1994; 14:7616–28. [PubMed: 7996200]
- Gomez TM, Letourneau PC. Filopodia initiate choices made by sensory neuron growth cones at laminin/fibronectin borders in vitro. *J Neurosci*. 1994; 14:5959–72. [PubMed: 7931556]
- Fawcett JW. The glial response to injury and its role in the inhibition of CNS repair. *Adv Exp Med Biol*. 2006; 557:11–24. [PubMed: 16955702]
- Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol*. 2008; 209:294–301. [PubMed: 17617407]
- Hill CE, Proschel C, Noble M, Mayer-Proschel M, Gensel JC, Beattie MS, Bresnahan JC. Acute transplantation of glial-restricted precursor cells into spinal cord contusion injuries: survival,

- differentiation, and effects on lesion environment and axonal regeneration. *Exp Neurol*. 2004; 190:289–310. [PubMed: 15530870]
- Hynds DL, Snow DM. Axon outgrowth inhibition by chondroitin sulfate proteoglycan: stalling/ stopping exceeds turning in human neuroblastoma growth cones. *Exp Neurol*. 1999; 160:244–55. [PubMed: 10630209]
- Hynds DL, Snow DM. Fibronectin and laminin elicit differential behaviors from SH-SY5Y growth cones contacting inhibitory chondroitin sulfate proteoglycans. *J Neurosci Res*. 2001; 66:630–42. [PubMed: 11746383]
- Jin Y, Neuhuber B, Singh A, Bouyer J, Lepore A, Bonner J, Himes T, Campanelli JT, Fischer I. Transplantation of human glial restricted progenitors and derived astrocytes into a contusion model of spinal cord injury. *J Neurotrauma*. 2011; 28:579–94. [PubMed: 21222572]
- Koprivica V, Cho KS, Park JB, Yiu G, Atwal J, Gore B, Kim JA, Lin E, Tessier-Lavigne M, Chen DF, He Z. EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans. *Science*. 2005; 310:106–110. [PubMed: 16210539]
- Jones LL, Margolis RU, Tuszynski MH. The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. *Exp Neurol*. 2003; 182:399–411. [PubMed: 12895450]
- Lelkes, PI.; Unsworth, BR.; Saporta, S.; Cameron, DF.; Gallo, G. Culture of cells for tissue engineering. In: Vunjak-Novakovic, G.; Freshney, RI., editors. *Culture of neuroendocrine and neuronal cells for tissue engineering*. Vol. Chap 14. Wiley; New York: 2006.
- Lepore AC, Han SS, Tyler-Polsz CJ, Cai J, Rao MS, Fischer I. Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. *Neuron Glia Biol*. 2004; 1:113–126. [PubMed: 16520830]
- Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Exp Neurol*. 2005; 194:230–242. [PubMed: 15899260]
- Lepore AC, Neuhuber B, Connors TM, Han SS, Liu Y, Daniels MP, Rao MS, Fischer I. Long-term fate of neural precursor cells following transplantation into developing and adult CNS. *Neuroscience*. 2006; 139:513–530. [PubMed: 16458439]
- Malla N, Berg E, Uhlin-Hansen L, Winberg JO. Interaction of pro-matrix metalloproteinase-9/ proteoglycan heteromer with gelatin and collagen. *J Biol Chem*. 2008; 283:13652–13665. [PubMed: 18359769]
- Matsui F, Oohira A. Proteoglycans and injury of the central nervous system. *Congenit Anom (Kyoto)*. 2004; 44:181–8. [PubMed: 15566408]
- Nout YS, Culp E, Schmidt MH, Tovar CA, Pröschel C, Mayer-Pröschel M, Noble MD, Beattie MS, Bresnahan JC. Glial restricted precursor cell transplant with cyclic adenosine monophosphate improved some autonomic functions but resulted in a reduced graft size after spinal cord contusion injury in rats. *Exp Neurol*. 2011; 227:159–71. [PubMed: 21040723]
- Properzi F, Asher RA, Fawcett JW. Chondroitin sulphate proteoglycans in the central nervous system: changes and synthesis after injury. *Biochem Soc Trans*. 2003; 31:335–336. [PubMed: 12653631]
- Properzi F, Carulli D, Asher RA, Muir E, Camargo LM, van Kuppevelt TH, ten Dam GB, Furukawa Y, Mikami T, Sugahara K, Toida T, Geller HM, Fawcett JW. Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. *Eur J Neurosci*. 2005; 21:378–90. [PubMed: 15673437]
- Rhodes KE, Fawcett JW. Chondroitin sulphate proteoglycans: preventing plasticity or protecting the CNS? *J Anat*. 2004; 204:33–48. [PubMed: 14690476]
- Sandvig A, Berry M, Barrett LB, Butt A, Logan A. Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. *Glia*. 2004; 46:225–251. [PubMed: 15048847]
- Schlessinger J, Lax I, Lemmon M. Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell*. 1995; 83:357–360. [PubMed: 8521464]
- Schmalfeldt M, Bandtlow CE, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR. Brain derived versican V2 is a potent inhibitor of axonal growth. *J Cell Sci*. 2000; 113:807–16. [PubMed: 10671370]

- See J, Bonner J, Neuhuber B, Fischer I. Axon outgrowth of neural progenitors in presence of inhibitory proteoglycans. *J of Neurotrauma*. 2010; 27:951–957. [PubMed: 20102265]
- Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, Liu K, He Z, Silver J, Flanagan JG. PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science*. 2009; 326:592–596. [PubMed: 19833921]
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci*. 2004; 5:146–156. [PubMed: 14735117]
- Snow DM, Letourneau PC. Axon outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J Neurobiol*. 1992; 23:322–36. [PubMed: 1624935]
- Snow DM, Mullins N, Hynds DL. Nervous system-derived chondroitin sulfate proteoglycans regulate growth cone morphology and inhibit axon outgrowth: a light, epifluorescence, and electron microscopy study. *Microsc Res Tech*. 2001; 54:273–86. [PubMed: 11514984]
- Snow DM, Smith JD, Cunningham AT, McFarlin J, Goshorn EC. Axon elongation on chondroitin sulfate proteoglycans is characterized by axonal fasciculation. *Exp Neurol*. 2003; 182:310–21. [PubMed: 12895442]
- Smith GM, Rutishauser U, Silver J, Miller RH. Maturation of astrocytes in vitro alters the extent and molecular basis of axon outgrowth. *Dev Biol*. 1990; 138:377–90. [PubMed: 2318341]
- Su YK, Elam JS. Proteoglycan regulation of goldfish retinal explant growth on optic tectal membranes. *Brain Res Dev Brain Res*. 2003; 142:169–175.
- Tan CL, Kwok JC, Patani R, Ffrench-Constant C, Chandran S, Fawcett JW. Integrin activation promotes axon growth on inhibitory chondroitin sulfate proteoglycans by enhancing integrin signaling. *J Neurosci*. 2011; 17:6289–95. [PubMed: 21525268]
- Tom VJ, Steinmetz MP, Miller JH, Doller CM, Silver J. Studies on the development and behavior of the dystrophic growth cone, the hallmark of regeneration failure, in an in vitro model of the glial scar and after spinal cord injury. *J Neurosci*. 2004; 24:6531–9. [PubMed: 15269264]
- Winberg JO, Kolset SO, Berg E, Uhlin-Hansen L. Macrophages secrete matrix metalloproteinase 9 covalently linked to the core protein of chondroitin sulphate proteoglycans. *J Mol Biol*. 2000; 304:669–680. [PubMed: 11099388]
- Yu P, Santiago LY, Katagiri Y, Geller HM. Myosin II activity regulates neurite outgrowth and guidance in response to chondroitin sulfate proteoglycans. *J Neurochem*. 2011
- Yu X, Bellamkonda RV. Dorsal root ganglia axon extension is inhibited by mechanical and chondroitin sulfate-rich interfaces. *J Neurosci Res*. 2001; 66:303–10. [PubMed: 11592128]
- Zuo J, Ferguson TA, Hernandez YJ, Stetler-Stevenson WG, Muir D. Neuronal matrix metalloproteinase-2 degrades and inactivates a neurite-inhibiting chondroitin sulfate proteoglycan. *J Neurosci*. 1998; 18:5203–11. [PubMed: 9651203]

Highlights

- NRP-derived axons cross borders between laminin and CSPGs
- NRP growth cones exhibit lower levels of PTP σ and LAR compared to sensory neurons
- NRP-derived axons cross onto CSPG independent of GRP contact and secreted factors
- GRPs secrete factors that promote axon outgrowth of sensory neurons onto CSPG
- GRP secreted factors decrease the inhibitory properties of the CSPG substrata

Figure 1. NRP-derived neurons extend axons across inhibitory proteoglycan barriers
(A) Examples of embryonic chick and rat sensory axons (TUJ1-green) turning at a CSPG border (100 μ g/mL; CS-56-red) (scale bar 100 μ m and 50 μ m respectively). **(B)** Various examples of NRP-derived neurons, co-cultured with GRP, extending axons (TUJ1-green) into inhibitory CSPG (100 μ g/mL; CS-56-red) (scale bar 20 μ m). **(C)** Quantification of the percentage of axons that cross CSPG border from rat NRP/GRP, chick DRG, and rat DRG in control medium (n = 19, 109, 97 respectively). On average, 78% of axons derived from NRP/GRP mix cultures crossed into the CSPG region compared to an average of 4% of sensory neurons (raw categorical data analyzed with χ^2 test, $p < 0.0001$ for NRP/GRP compared to both rat and chick DRG).

Figure 2. NRP-derived neurons that cross into CSPG are not directionally biased and their axon lengths are not affected by CSPG

(A) Schematic of analysis to determine if the neurites crossing into CSPG are directionally biased. The cell is divided into four quadrants and the direction of neurite extension relative to the CSPG border is recorded. (B) Quantification of the direction at which the neurite extends from the cell body relative to the CSPG border. There was no difference in the direction of neurite extension ($p = 0.6$, X^2 test performed on raw categorical data, $n = 63$). (C) Examples of NRP-derived neurons (TUJ1-green) positioned on LN or CSPG (100 µg/mL; CS-56-red) (scale bar 15 µm). (D) Quantification of neurite length of NRP-derived neurons on LN (average length 158 µm ± 11) and CSPG (average length 148 µm ± 12) (Data are mean ± SEM, Mann-Whitney test $p = 0.3946$, $n = 34, 40$ respectively).

Figure 3. NRP-derived neurons show reduced levels of PTPσ staining

(A) Examples of growth cones from rat DRG, chick DRG, and rat NRP/GRP-derived neurons. All cultures were plated on PL/LN coated coverslips and images were acquired with a 100x objective (scale bar 15μm). Note the punctate staining of PTPσ, and the localization of PTPσ throughout growth cone and filopodia. Omission of the primary antibody resulted in undetectable levels of staining at image acquisition parameters that yielded strong signal from samples stained with the primary antibody. (B) Quantification of total PTPσ content in growth cone of embryonic day 19 DRG neurons and embryonic day 13.5 NRP/GRP neurons (n = 29, 30 respectively). NRP-derived neurons show a 74% decrease in total staining levels for PTPσ in growth cones compared to neurons of DRG (Data are mean ± SEM, Mann-Whitney test, p = 0.0001).

Figure 4. NRP-derived neurons show reduced levels of LAR staining

(A) Examples of growth cones from rat DRG, chick DRG, and rat NRP/GRP-derived neurons. All cultures were plated on PL/LN coated coverslips and images were acquired with a 100x objective (scale bar 15 μ m). (B) Quantification of total LAR content in growth cone of embryonic day 19 DRG neurons and embryonic day 13.5 NRP/GRP neurons ($n = 26, 26$ respectively). NRP-derived neurons show a 95% decrease in total LAR staining levels in growth cones compared to neurons of DRG (Data are mean $\pm$ SEM, Mann-Whitney test, $p = 0.0001$).

Figure 5. The ability of NRP axons to cross onto CSPG is independent of GRP secreted factors (A) Examples of axons (TUJ1-green) of embryonic rat and chick sensory neurons turning at a CSPG border (CS-56-red) cultured with GRP-conditioned medium (scale bar 50 μ m and 100 μ m respectively). Compare images to Figure 1A in control medium. (B) Quantification of the percentage of DRG axons that cross onto CSPGs when cultured with control medium, NRP/GRP-conditioned medium, or GRP-conditioned medium (rat DRG axons, n = 227, 122, 196; chick DRG axons, n = 110, 100, 100 respectively). There was no difference between the average number of axons that crossed onto the CSPG between control and either of the conditioned mediums, as represented in bar graph (raw categorical data analyzed with χ^2 test, $p > 0.05$ for all groups; rat and chick).

Figure 6. Conditioned medium from high density GRP cell cultures lessens the inhibitory response of DRG axons to CSPG

(A) Examples of embryonic rat sensory neurons (TUJ1-green) turning at a CSPG border (CS-56-red) cultured with control medium or GRP-HDCM. (B) Quantification of the percentage of DRG axons that cross onto CSPG cultured with different concentrations of GRP-HDCM. GRP-conditioned medium has a dose dependent effect on the response by DRG axons to CSPG. Axons of rat DRG cultured with 100% or a dilution of 50% GRP-HDCM are more likely to cross onto the CSPG compared to axons cultured with a dilution containing 10% GRP-HDCM or control medium (percentage of axons to cross onto CSPG relative to the concentration of GRP-HDCM; 0 %HDCM = 4%, 10%HDCM= 3%, 50%HDCM = 8%, 100%HDCM = 16%; # of axons n = 234, 203, 217, 295 respectively) (raw categorical data analyzed with χ^2 test and compared to control, 10% p = 0.8, 50% p = 0.037, 100% p < 0.0001). (C, E) Examples of rat DRG neurons (TUJ1-green) attached and extending axons on CSPG (CS-56-red) cultured with control medium or GRP-HDCM. (D) Comparison of the percentage of DRG neurons on CSPG that extend axons when cultured in different concentrations of GRP-HDCM. The percentage of DRG neurons that extend axons on CSPG increases significantly with increased concentrations of GRP-HDCM (percentage of neurons to extend axons on CSPG relative to the concentration of GRP-HDCM; 0 %HDCM = 39%, 10%HDCM= 55%, 50%HDCM = 68%, 100%HDCM = 76%; # of neurons n = 156, 233, 163, 120 respectively) (raw categorical data analyzed with χ^2 test and compared to control, 10% p = 0.0015, 50% and 100% p < 0.0001). (F) Quantification of the length of DRG axons extending on CSPG cultured with different concentrations of GRP-HDCM. The length of DRG axons extending on CSPG is dependent on the concentration of GRP-HDCM compared to control (length of axons extending on CSPG relative to the

concentration of GRP-HDCM; 0 % = $28\mu\text{m} \pm 3$, 10% = $44\mu\text{m} \pm 3$, 50% = $57\mu\text{m} \pm 3$, 100% = $75\mu\text{m} \pm 3$; # of axons n = 163, 190, 279, 359 respectively) (Data are mean $\pm$ SEM, Mann-Whitney test, all conditions compared to control, 10% p = 0.0007, 50% and 100% p < 0.0001). (Scale bar = 20 μm for all images)

Figure 7. Pre-treatment with conditioned medium from high density GRP cell cultures lessens the inhibitory response of DRG axons to CSPG

(A) Examples of embryonic rat sensory neurons (TUJ1-green) turning at a CSPG border (CS-56-red) that were pre-treated for 24hrs with control medium or GRP-HDCM. (B) Quantification of the percentage of DRG axons that cross onto CSPG following pre-treatment of substratum with control medium or GRP-HDCM. Pre-treatment of substratum with GRP-HDCM increased the percentage of rat DRG axons to cross onto CSPG (23%) compared to pre-treatment with control medium (3%) (# of axons $n = 591, 894$ respectively; raw categorical data analyzed with X^2 test $p < 0.0001$). (C, E) Examples of rat DRG neurons (TUJ1-green) attached and extending axons on CSPG (CS-56-red) substratum pre-treated with control medium or GRP-HDCM. (D) Quantification of the percentage of DRG neurons on CSPG following pre-treatment of substratum with control medium or GRP-HDCM. The percentage of DRG neurons that extend axons on CSPG increased significantly with 24hr pre-treatment of substratum with GRP-HDCM (90%) compared to control (45%) (# of neurons $n = 482, 360$ respectively; raw categorical data analyzed with X^2 test $p < 0.0001$). (F) Quantification of the length of DRG axons extending on CSPG following pre-treatment of substratum with control medium or GRP-HDCM. The average length of DRG axons extending on CSPG increased significantly in cultures pre-treated with GRP-HDCM ($132\mu\text{m} \pm 8$) compared to control ($58\mu\text{m} \pm 5$) (# of axons $n = 87, 171$ respectively; data are mean $\pm$ SEM, Mann-Whitney test, $p < 0.0001$). (Scale bar = $20\mu\text{m}$ for all images)