2016

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Research article

Role of the Chondroitin Sulfate Proteoglycan, Neurocan, in Inhibition of Sensory Neurite Regeneration

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Abbreviated Title: *Role of Neurocan in Inhibition of Sensory Neurite Regeneration*

Pages: 13

Figures: 5

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Key Words: Astrocytes, Chick, Rat, TGF-β, Sensory Neuron, Co-Culture

Introduction

In the adult mammalian brain and spinal cord, neuronal injury results in failed neurite regeneration, in part due to the up-regulation of chondroitin sulfate proteoglycans (CSPGs).[1,9] CSPGs are molecules consisting of a protein core with covalently bound glycosaminoglycans (GAGs), specifically, chondroitin sulfate side-chains. The majority of CSPGs produced after injury originate from reactive astrocytes found in the glial scar surrounding the injury site.[1] Although this milieu is very complex and involves more than just CSPGs, axonal regrowth may be improved if the expression of specific, highly inhibitory CSPGs produced after injury were attenuated selectively.[1] Specifically, the goal is to attenuate the inhibitory part of the CSPG, while minimizing interference with the beneficial components of the CSPG.[2,7] Neurocan (Figure 1) is one type of CSPG that is upregulated after injury and inhibits neurite regeneration.[1,7]

The over-arching goal of this study was to determine the role of the CSPG, neurocan, in inhibition of sensory neurite regeneration. To determine this, chicken neurons were co-cultured with chicken astrocytes, focusing on two experimental protocols. First the conditions for growth of chick astrocytes were optimized, since previous studies used rat astrocytes and a variety of growth conditions. Second, a comparison was made of the response of sensory neurons in both the presence of astrocytes that express neurocan, and those in which neurocan was "knocked down" by shRNA transfection.

In order to optimize the conditions for chick astrocyte growth, a series of preliminary tests was performed on chick astrocytes. Initially, to confirm the identity of the harvested cells as astrocytes. it was shown that the chick astrocytes express Glial

Fibrillary Acidic Protein (GFAP) in culture.[10] GFAP recognizes reactive astrocytes, i.e. those that typically express CSPGs.[10] Additionally, an experiment was performed to monitor the upregulation of CSPGs after treatment with Transforming Growth Factor-β (TGF-β), previously shown to mimic injury *in vitro* in rat astrocytes[3]. Overall, preliminary co-culture studies with both chick and rat astrocytes suggest the "knockdown" of CSPGs may render astrocytes more permissive for axonal outgrowth, and potentially, a better substratum for regeneration following spinal cord injury.

Methods

Cell Culture:

Circular glass coverslips (25mm) were prepared for tissue culture by incubation with 2 ml of 0.1mg/ml Poly-L-Ornithine (PLO) at 37°C for 10 min., a substratum that promotes neuron outgrowth *in vivo*.[4] The coverslips were then washed 3x with sterile water and dried overnight.

Astrocyte Preparation

Embryonic chicken day 17-18 brains or post-natal rat day 1-2 brains were harvested and enzymatically dissociated. Cells were cultured for 24 hr. This conditioned media was supplemented with 50% fresh DMEM. Subsequent media changes (100% fresh media) were performed every 3 days, as necessary. At 75% confluency, cells were gently shaken on a Thermoscientific Shaker for 8 hr. followed by a final media change. The resulting pure population of astrocytes was cultured for 24 hr. and then split into separate culture flasks (1:4) for future use.

Preparation of Reactive Astrocytes

Each well in a 24-well tissue culture plate was seeded with 18,000 astrocytes, the necessary number to reach 15-20% confluency within 2 days. The media was replaced with fresh media when the astrocytes reached 5-10% confluency. At 15-20% confluency, 5 ng/ml the growth factor, TGF-β, was added to each well to induce a chemical injury and promote a reactive astrocyte phenotype .[3] Controls did not receive TGF-β. The TGF-β remained in the wells for 48 hr. (for the preliminary TGF-β test protocol, see "Cell Fixation".)

Transfection

A dilution was performed for both neurocan shRNA #1 and Green Fluorescent Protein (GFP) negative control. Neurocan #1 DNA (170μL at 0.89 mg/ml) was diluted in 1,515μL serum-free Astrofeed, and 153 μL of GFP negative control DNA (0.99 mg/ml) was diluted in 1,515 μL serum-free Astrofeed. Each individual dilution was then mixed separately with a dilution of 151.5 μL lipofectamine 2K in 1,515 μL serum-free Astrofeed and incubated at 37°C for 5 min. Neurocan shRNA #1/lipofectamine mixture (300μL) was added to 3 wells and 300 μL of the GFP negative control/lipofectamine mixture was added to a separate 3 wells for each trial. The cultures were incubated overnight at 37°C.

Dorsal Root Ganglion (DRG; Sensory Neuron) Dissection

Embryonic chicken day 8-10 dorsal root ganglion neurons (DRGs) were harvested and enzymatically dissociated. Neuronal populations were purified by a 3 hr panning step.[8] DRGs (20,000) were then cultured for 24 hr with the transfected astrocytes at 37°C.

Cell Fixation

Cells were fixed with 2 ml/well 4% paraformaldehyde and 4% sucrose in 0.1M (1x) PBS for 10 min. at 37°C. Fixative was removed and the cells were washed 3x with 1x PBS. The cells were covered and stored at 4°C.

Blocking, Antibody Incubation, & Mounting

The cells were permeabilized for 10 min at room temperature with 0.25% Triton-X100 in 1x PBS, then blocked for 1 hr at 37°C in 1x 10% PBS/BSA. The cells were then incubated overnight at 4°C with 1° antibody diluted 1:4000 in 1x PBS/3% BSA. They were then washed 5x with 1x PBS for 5 min followed by incubation with the 2° antibody diluted 1:1000 in 1x PBS/3% BSA for 1-2 hr in the dark at 37° C. Cells were again washed 5x with 1x PBS for 5 min. The coverslips were then mounted with Fluromount-G, 2 coverslips/slide and dried for 2 days before visualization. For conditions using TGF-β to mimic injury in vitro, an anti-chondroitin sulfate antibody, CS-56, was used, followed by a donkey anti-mouse IgG Alexafluor 488 2° antibody. Reactive astrocytes were identified using an anti-GFAP 1° antibody and donkey anti-mouse IgG Alexafluor 488 2° antibody. Neurons in the co-culture were visualized using an anti-βIII-tubulin 1° antibody and donkey anti-rabbit IgG Alexafluor 555 2° antibody.

Results

Rat and chicken astrocytes were cultured to 1) optimize growth conditions for chicken astrocytes, and 2) compare and contrast requirements for the culture and growth of chick astrocytes compared to rat, since previous studies relied solely on culture of rat astrocytes. However, in the present study, since chick neurons were the test cell type, it was deemed advantageous to perform the experiments with species-specific conditions. Both rat (Fig. 2A) and chick (Fig. 2B) cortical astrocytes expressed the marker, glial fibrillary acidic protein, or GFAP. This marker was used to identify reactive astrocytes in tissue culture. Importantly, the expression of GFAP, indicating that astrocytes are reactive, typically correlates with an upregulation of CSPGs.[1]

The test to monitor the upregulation of CSPGs after treatment with Transforming Growth Factor-β (TGF-β), which mimics injury *in vitro*, demonstrated that chick astrocytes (Fig. 3) follow this pattern of injury *in-vitro*, similar to rat astrocytes (Fig. 4). Specifically, cortical rat and chick astrocytes were either treated or untreated with TGF-β. Immunoreactivity using antibody CS-56 indicated the expression and upregulation of CSPGs in the TGF-β treated neurons.[3] Thus, as predicted, TGF- β mimicked injury in rat and chick astrocytes. Astrocytes treated with TGF-β, like those *in vivo* following spinal cord injury, upregulate many CSPGs and are inhibitory to neuronal outgrowth *in vitro*. Given that TGF-β upregulated CSPGs in chick astrocytes, the next step for this study will be to repeat co-culture analyses using chick astrocytes with chick DRG neurons, according to the same protocol used previously for rat astrocytes.

To test the response of chick DRG neurons to treated astrocytes, a co-culture experiment was done using cortical rat astrocytes and chick DRG (sensory) neurons (Fig.

5). DRG axons grew on and over rat astrocytes that were GFP+, indicating a reduction in inhibition. Not surprisingly, the site where the axon is growing over the astrocyte in this case is very lowly fluorescent, indicating low neurocan levels, and would account for the lack of inhibition seen only at this location along the astrocyte (Fig. 5).

In all, these data confirm that neurocan expressed by rat and chick astrocytes is inhibitory to DRG neuron outgrowth and regeneration. Further, the data indicate that neurocan knock down, in spite of the presence of other CSPGs, can promote axon outgrowth.

Discussion

The overall goal of the study was to focus specifically on the response and growth of sensory neurons in the presence of astrocytes that express neurocan, in relation to those astrocytes in which neurocan has been "knocked down" by shRNA transfection. The preliminary experiments performed to optimize conditions for the co-culture experiment confirmed that chick astrocytes express the astrocyte marker, GFAP, indicating successful re-creation of an injury response in vitro, as has been shown previously.[7] Neurons express GFAP when challenged in this way, and concomitantly express CSPGs that result in inhibition of neural regeneration. Specifically, we showed that chick DRG sensory neurons upregulate inhibitory CSPGs upon treatment with TGF-β, similar to that shown for the rat model.[3] Importantly, when one of these CSPGs, neurocan, is eliminated or reduced, DRG neurons are competent to extend axons across the neuron-free regions of the cell, demonstrating a role for neurocan in inhibition (See Fig. 5).

While minimal, these tests have laid the groundwork for future experiments using chick astrocytes in place of rat astrocytes in the co-culture experiment with chick dorsal root ganglia, in order to provide species specific comparisons in these analyses.

Future Directions

Future studies will use the newly optimized chick astrocytes to repeat and expand upon the co-culture experiments previously performed with rat astrocytes. The purpose of optimizing the co-culture paradigm is to be able to evaluate neuronal responses to neurocan using species specific cell types (i.e. chick astrocytes with chick neurons). Axonal outgrowth will be quantified according to a clear set of guidelines, allowing determination of the contribution of the CSPG, neurocan, and subsequently other CSPGs, to improved regeneration.

While our hypothesis is that shRNA transfection for neurocan, i.e. decreased expression of neurocan, will render the astrocytes more permissive to axonal outgrowth in general, quantification will be needed verify this response. The interactions between neurons and astrocytes will be counted according to the following criteria: 1) the cell body cannot be on the astrocyte, 2) the neurons must have one, and only one, interaction with the surface of a transfected astrocyte, and 3) if there are multiple interactions, only the first interaction will be counted. Using these stringent criteria, we will determine the role of neurocan in DRG neurite inhibition and regeneration in the chick model.

We expect that control astrocytes will exhibit inhibitory properties and neurons will avoid growing over them, and/or will turn with contact.[1,2,6,7] These results would be

consistent with previous studies performed such as one that used the CSPG aggrecan and a stripe assay to measure sensory neuron growth cone and filopodial response in the presence of aggrecan variant.[6] This study demonstrated that although neurite outgrowth varies with differences in aggrecan structure, generally it is inhibitory to neurons.[6]

Overall, preliminary co-culture studies with both chick and rat astrocytes suggest the "knockdown" of CSPGs may render astrocytes more permissive for axonal outgrowth, and potentially, regeneration following spinal cord injury.[1,2,6] This hypothesis is consistent with previous literature, however, it aims to elucidate the success of a possible mechanism to knock down the specific CSPG neurocan. The co-culture experiment and sensory neurite growth analysis will thus further supplement previous studies, while evaluating the role of shRNA transfections as a means to inhibit inhibitory CSPGs.

Acknowledgements

The authors wish to acknowledge the support of the NIH (NINDS, NS054370) to DMS, and the Run2Walk Foundation to DMS, and graphic design assistance from Aidan Shafran.

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Figures

Figure 1

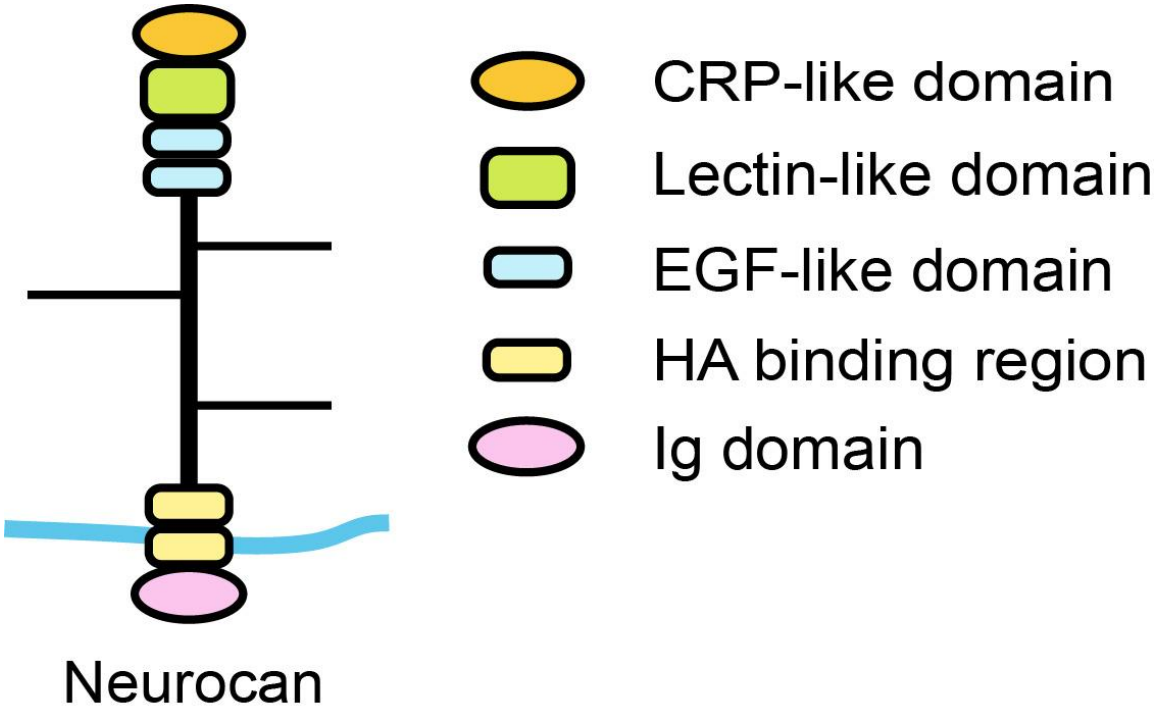

Figure 2

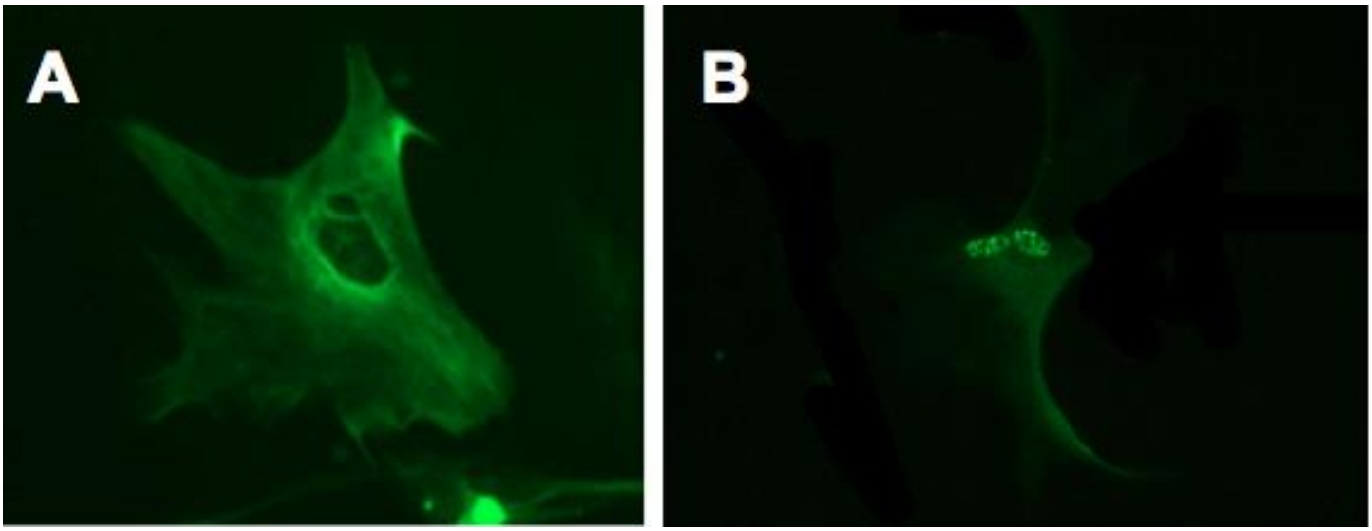

Figure 3

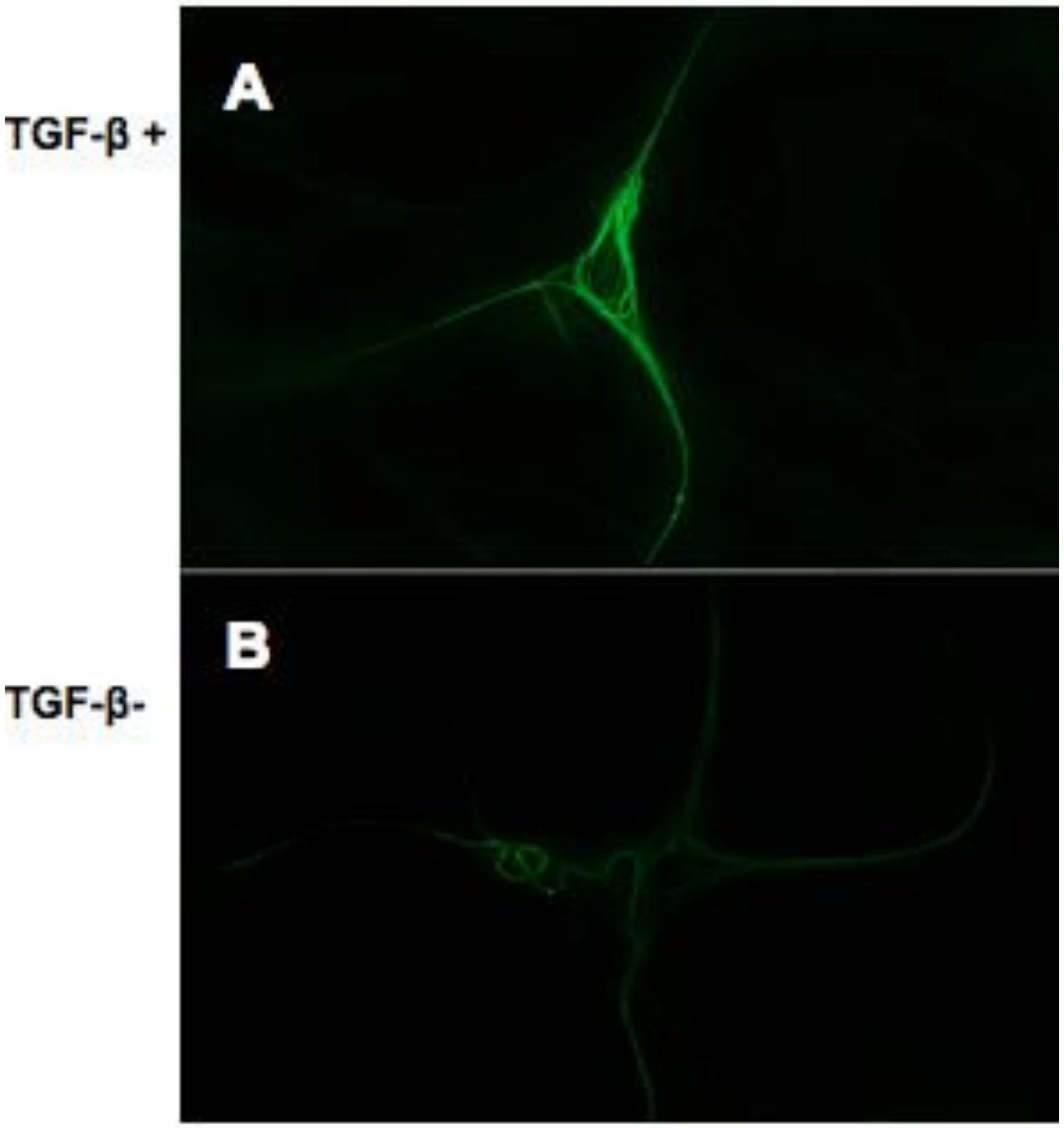

Figure 4

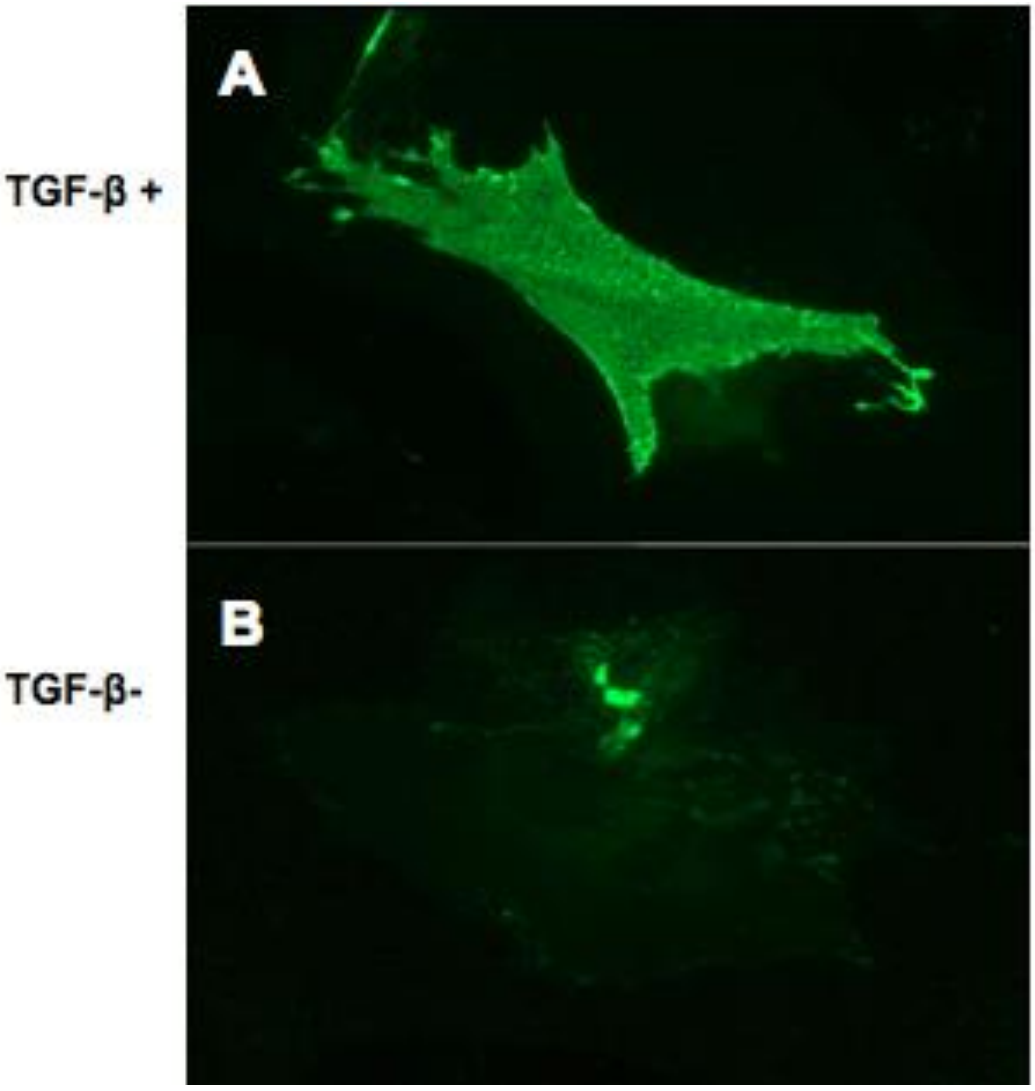

Figure 5

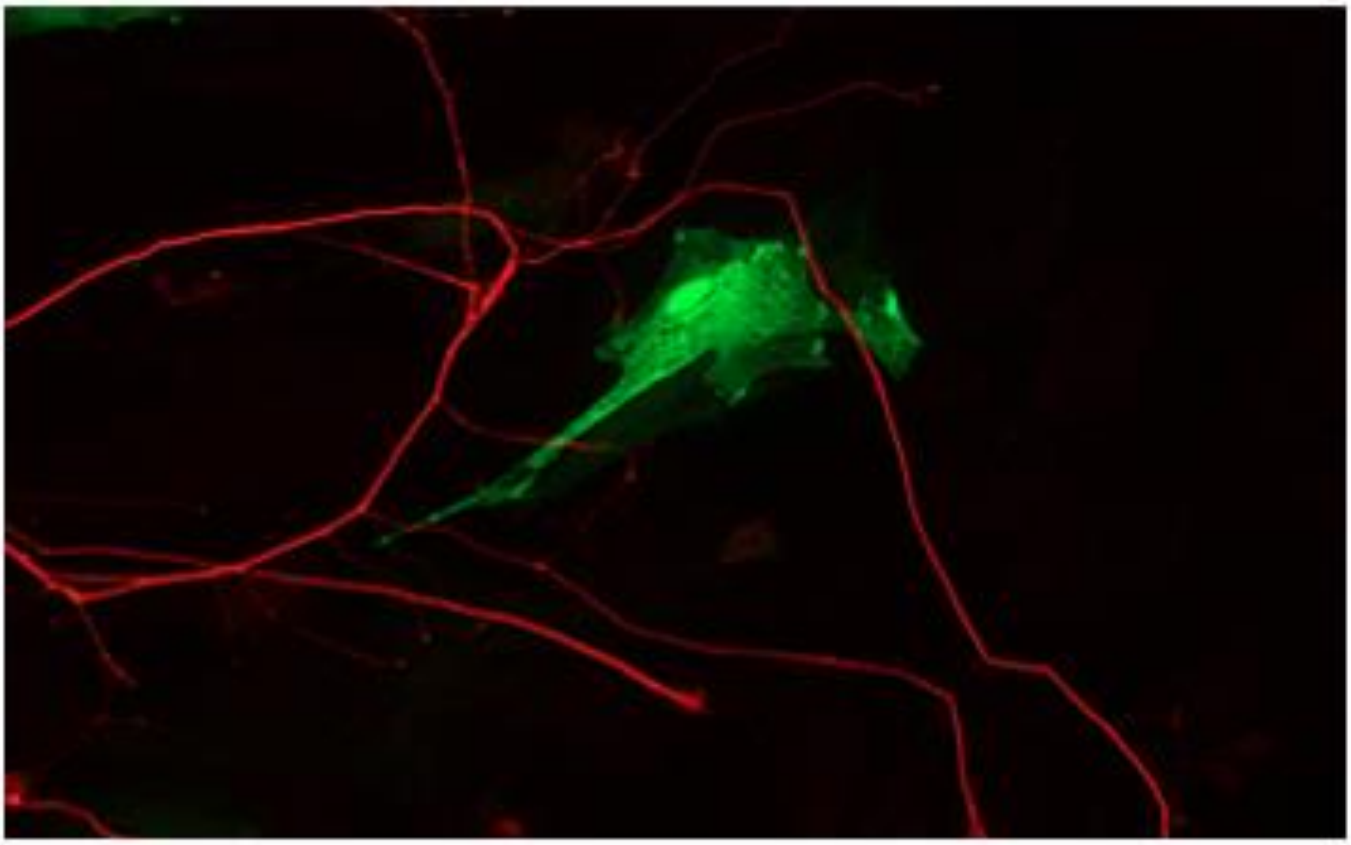

Figure Legends

Figure 1. *Neurocan-Structural models of chondroitin sulfate proteoglycans*- (A) Lectican family members (aggrecan, versican, neurocan and brevican) consist of an N-terminal G1 domain, which binds with hyaluronic acid, a chondroitin sulfate (CS) attachment region and a C-terminal G3 domain.[5]

Figure 2. *GFAP Labeled Chick & Rat Astrocytes*- Both rat (A) and chick (B) cortical astrocytes express the marker, glial fibrillary acidic protein, or GFAP. This test served to identify astrocytes in tissue culture.

Figure 3. *Rat astrocytes in the presence or absence of TGF-β*- Cortical rat astrocytes were either treated (TGF-β+; Fig. 3A), or untreated (TGF-β-; Fig. 3B). Fluorescence indicates the expression and upregulation of CSPGs in the TGF-β treated wells (to mimic injury). Astrocytes treated with TGF-β, like those *in vivo* following spinal cord injury, are inhibitory to neuronal outgrowth *in vitro*.

Figure 4. *Chick astrocytes in the presence or absence of TGF-β*- Cortical chick astrocytes were either treated (TGF-β+; Fig. 4A), or untreated (TGF-β-; Fig. 4B). Fluorescence indicates the expression and upregulation of CSPGs in the TGF-β treated wells (to mimic injury). Astrocytes treated with TGF-β, like those *in vivo* following spinal cord injury, are inhibitory to neuronal outgrowth *in vitro*.

Figure 5. *Rat Co-Culture*- Illustration of a cortical rat astrocyte co-cultured with chick DRG (sensory) neurons. DRG axons grow on and over rat astrocytes that are GFP+, indicating neurocan knock-down.