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Do Sensory Neurons Secrete an Anti-Inhibitory Factor that Promotes Regeneration?

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Introduction

Spinal Cord Injury (SCI) is a devastating and debilitating condition. According to a recent US survey (<https://www.nscisc.uab.edu>), currently there is an average of 270,000 persons living with SCI, and new injuries occur at a rate of about 12,000 cases per year. This condition presents formidable challenges to successful post-trauma recovery of function, e.g. since neurons in the adult CNS do not regenerate following injury, permanent conditions such as paralysis can result.

One hallmark of regeneration failure following SCI is the up-regulation of inhibitory molecules, chondroitin sulfate proteoglycans (CSPGs), secreted by reactive astrocytes of the glial scar. CSPGs are large aggregating macromolecules consisting of a core protein to which glycosaminoglycans (GAGs) are covalently bound. While the structures of the GAG chains differ between CSPGs, the length, amount and arrangement of the GAGs are known to contribute to inhibition of axonal outgrowth associated with SCI. Aggrecan is one type of CSPG that inhibits regeneration and blocks recovery of function. Data in vitro and in vivo show removal of aggrecan GAGs can lead to successful axonal outgrowth. Might there be other ways to also stimulate outgrowth even in the presence of GAGs?

The present study is based on preliminary data showing if sensory neuron explants are plated on either side of an aggrecan-adsorbed region, the typically inhibitory nature of aggrecan is attenuated and an unprecedented level of outgrowth can occur across the inhibitory region. Thus, here we formally tested the ability of sensory neurons to lure other sensory neurons to regenerate in spite of the presence of an inhibitory aggrecan. From these results, we now hypothesize that sensory explants overcome inhibitory CSPGs by secreting an axon growth-inducing factor to promote regeneration of injured neurons.

Methods

Patterned substratum: Glass coverslips were mounted over holes in the bottom of 50 mm polystyrene Petri dishes to create a small glass-bottom culture surface for excellent optics. The coverslips were coated with poly-D-lysine (PDL) to bind proteins of interest. For controls, Whatman filter paper rectangles (1 cm x 350 μ m) were soaked in laminin (25 μ g/ml; EHS laminin, BD Biosciences) and adsorbed to the PDL surface. When dry, the paper was removed and the entire coverslip was coated with laminin. This created a laminin:laminin choice assay. For test cultures, filter paper rectangles were soaked in 10 μ l of aggrecan [200 μ g/ml; source]. When dry, the paper strips were removed and the

entire coverslip was coated with laminin (25 µg/ml), creating a choice assay of laminin:aggrecan, i.e. a laminin background with CSPG/laminin stripes; 2-3 per dish.

Tissue culture: Dorsal root ganglia (DRG) were dissected from White Leghorn embryonic chicken (E8-12 days) according to AAALAC regulations, adhering to an approved protocol. DRG were cleaned of surrounding connective tissue and cut into four small “explants” (consisting of DRG sensory neurons and non-neuronal cells).

The explants were plated using one of two conditions: 1) one explant on the left side only of the aggrecan-adsorbed stripe (termed “1:0”), or 2) one explant on each side (both left and right; termed “1:1”) of the aggrecan-adsorbed stripe. In all cases, explants were placed equidistant from the aggrecan stripe. The explants were cultured in a 5% CO₂ humidified incubator for 48 hrs at 37°C. The cultures were then washed in phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde.

Immunocytochemistry: The cultures were incubated in Goldberg Antibody Blocking Buffer for 1 hour. Neuron-specific beta-tubulin was visualized using a β-Tubulin III primary antibody (Sigma-Aldrich, 1:000), and subsequently reacted with an Alexa Fluor 488 (donkey anti-rabbit) secondary antibody (Invitrogen, 1:100). The aggrecan adsorbed in a striped pattern was visualized using an anti-chondroitin sulfate (clone CS-56 primary antibody; Sigma-Aldrich, 1:500), followed by a TRITC labeled goat anti-mouse IgM secondary antibody (Jackson ImmunoResearch Laboratories, Inc., 1:500). The plates were imaged on an epifluorescence microscope. The density of neurite outgrowth from the explants was quantified using a “midline crossover test”, in which the vertical midline of the aggrecan stripe was determined and the number of axons that crossed that boundary (crossovers) was recorded.

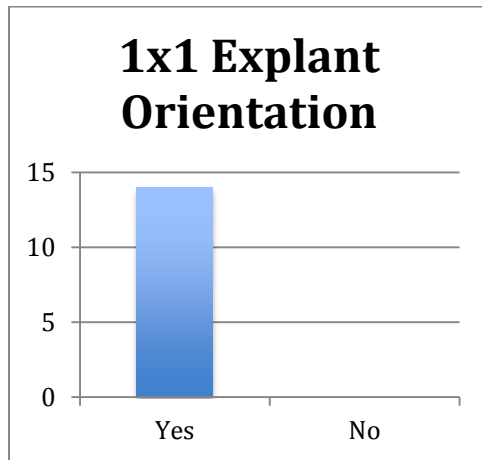
Results

From preliminary observations, it appeared that neurons were sometimes able to grow across inhibitory CSPGs, e.g. aggrecan, when explants were plated on either side of the aggrecan-adsorbed region, while not being able to do so if only one explant existed. This led to the notion that sensory neuron explants may produce an anti-inhibition factor. We set out to quantify this preliminary observation.

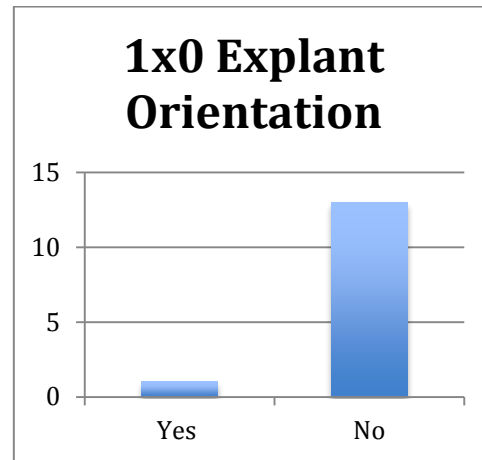
From data examining both single explants and explants on either side of an aggrecan stripe, we show here that sensory neurons were significantly more likely to cross a CSPG stripe if there was an explant opposed by another across the stripe (**Figure 1 A, B**). To quantify this behavior, the plate was scored as “Yes” if crossover occurred when an explant was present on each side of the stripe; and plates were scored as “No”, if axons were not able to grow onto the stripe when there was only one unopposed explant on one side of the stripe. Thus, the presence of a nearby explant can induce outgrowth across a typically inhibitory CSPG-adsorbed substratum.

Figure 1

A



B



Using immunocytochemistry to identify the position of the CSPG stripe (red) and the axons of DRG neurons (green), the images below show one representative result for each condition (1:0 and 1:1).

Figure 2A shows a merged image taken with the Zeiss Axiovision image analysis system. 1 x 0 explant orientation.

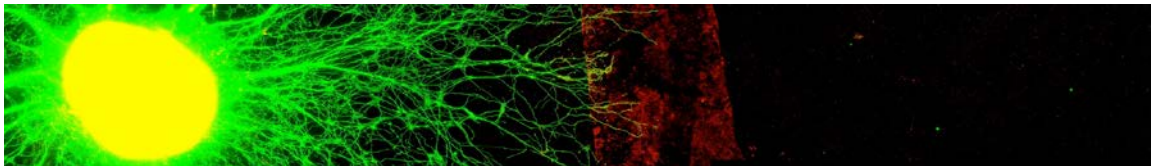
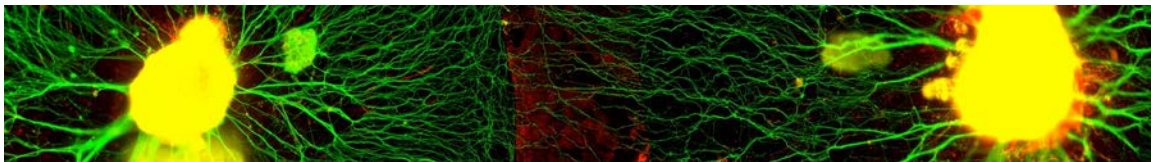
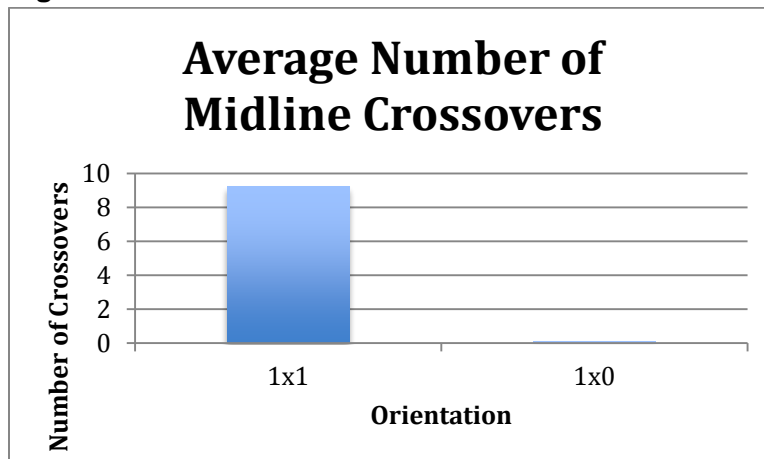


Figure 2B shows a merged image taken with the Zeiss Axiovision image analysis system. 1 x 1 explant orientation.



For each of the experiments, the average number of midline crossovers was determined. The average number of crossovers for the 1x0 orientation was 0.0714 crossovers per experiment, and 9.21 crossovers per experiment for the 1x1 orientation (n=14) (**Figure 3**).

Figure 3



Statistics: Using a Student's T-test, the results represent a significant difference ($p < 0.0001$) in the amount of sensory neuron outgrowth dependent upon the orientation in which they were placed in relation to the aggrecan stained stripe, i.e. 1:0 or 1:1.

Conclusions

Regeneration of sensory neurons following SCI is a major goal. To promote regeneration of injured neurons, we must first understand the environment in which injured neurons are elongating, and the capacity for outgrowth injured neurons possess. The present study demonstrated the proximity of DRG neurons to one another in a regeneration model in vitro significantly affects their ability to elongate, across an inhibitory terrain (aggrecan; CSPG). The data are consistent with the notion that sensory neurons may secrete an anti-inhibitory factor that promotes axonal outgrowth in the presence of a typically inhibitory molecule such as aggrecan (CSPG). Identification and purification of such an anti-inhibition factor could provide an important therapy for the treatment of SCI. Thus, the future goal of this project is the pursuit of this factor(s) using biochemical and molecular biological tools, and to test the factor(s) in models of SCI in vitro and in vivo.

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