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Behavioral and Morphological Parameters of Neurons Predict the Inhibitory Potential of CSPG Motifs: A Novel Technique

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Abstract

Our lab aims to systematically identify the structural elements of chondroitin sulfate proteoglycans (CSPGs) that inhibit regeneration following spinal cord injury (SCI). CSPGs are extracellular matrix molecules produced by astrocytes of glial scar tissue following SCI. Central to this project is the use of CSPGs referred to as “Designer PGs,” which contain engineered modifications in the GAG chains and/or the protein core of the neural CSPG aggrecan. Using established bioassays in vitro, we qualitatively and quantitatively measure growth cone behaviors and morphology as they interact with Designer PG molecules. These measurements are then translated into a composite inhibitory quotient (IQ) score that reflects the inhibitory strength of the particular Designer PG. Scoring is completed through application of our comprehensive inhibitory quotient (IQ) system scoring criteria. The utility of the IQ system is that it allows us to evaluate and directly compare all experimental CSPGs. IQ scores can then be mapped back to the structure of the experimental CSPG, allowing us to pinpoint the inhibitory domains of CSPGs. The long term goal of this methodology is to develop clinical therapies that selectively target the most inhibitory CSPG domains while leaving unaltered the beneficial aspects of the glial scar, thereby supporting regeneration and recovery of function following SCI.

Behavioral and Morphological Parameters of Neurons Predict the Inhibitory Potential of CSPG Motifs: A Novel Technique.

Faculty Mentor: Dr. Diane Snow

Eddie Kobraei is an exceptional student who has done exceptional research as a Beckman Scholar (2007-2008). His project in my lab entitled, “Quantification of Neuronal Growth Cone Responses to Specific Chondroitin Sulfate Proteoglycan Motifs” has been extremely challenging and represents a mature endeavor, even for more advanced students. Eddie has been diligent from day one and has done an amazing job, accumulating data, critically analyzing the data, and presenting the results at a variety of meetings both local and national. From observing Eddie as he progresses through this study, it is clear that he is very intelligent, highly-motivated, mature beyond his years, and capable. I have been very impressed with Eddie’s work ethic and drive as he has analyzed neuronal growth cone behavior in response to inhibitory proteoglycans. This project requires great attention to detail, and extreme accuracy, as well as long hours of analysis. Eddie has given 100% on all accounts. The article he is currently submitting to Kaleidoscope is written superbly and is a thorough representation of the project he has done. The depth and breadth of inquiry he describes demonstrate his integral involvement in the project and his abundant academic talents.
Introduction
Spinal cord injury (SCI) is a debilitating condition currently afflicting approximately 255,000 people in the U.S. alone (NSCISC, 2008). This condition presents formidable challenges to successful post-trauma recovery of function and often results in permanent central nervous system (CNS) damage. A hallmark of regeneration failure following SCI is the up-regulation of inhibitory chondroitin sulfate proteoglycans (CSPGs) by astrocytes of the glial scar (Fitch and Silver, 2008). One member of the CSPG family that is up-regulated is aggrecan (Figure 1), which can serve as a model for this family of aggregating proteoglycans. Although studies in vitro and in vivo have localized the inhibition largely to the glycosaminoglycan (GAG; sugar) portion of CSPGs (Snow et al., 1990), the specific parts of the GAG chains or possible protein domains responsible for inhibition remain unidentified. Further, the effects of specific CSPG motifs on neuronal growth cone behaviors have yet to be systematically quantified.

In the present study, we are identifying the precise structural microheterogeneities of CSPGs that make them inhibitory to neuronal growth cones, using a novel technique involving: 1) “Designer PGs” and, 2) an “Inhibitory Quotient (IQ)” analysis system. Designer PGs are discrete aggrecan mutants that are produced with altered properties (elimination of specific sites for glycosylation, alterations in sugar chain length, or number, composition, or deletion of individual domains) to identify modifications affecting neurite outgrowth. With the IQ system, a wide variety of behavioral and morphological parameters of neuronal growth cones are qualitatively and quantitatively evaluated from time-lapse images as growth cones contact and respond to Designer PGs. The IQ system is then used to translate these responses into composite IQ scores for each Designer PG, which is an indication of the degree of inhibitory strength of each PG (see Methods).

The results of this on-going study are serving to identify the structural moieties responsible for the inhibitory action of CSPGs, and are providing insight into the ways in which growth cones respond to proteoglycan molecules. The long-term goal of these studies is to develop strategies to manipulate the most inhibitory domains of CSPGs to render them non-inhibitory. In this way, glial scars will remain intact to facilitate the normal healing and repair processes, but CSPGs will allow regeneration, ultimately promoting recovery of function in patients with SCI.

Methods:
Bioassays
Sensory Neuron (DRG) Dissection
Embryonic dorsal root ganglia (DRG) neurons were dissected on ice according to AAALAC regulations governing animal welfare and transferred to culture dishes containing 500 µl of Dulbecco’s Modified Eagle Media (DMEM) with F12 HEPES in a 1:1 ratio. The DRG were cut into small explants and plated on the substratum described below. The substratum plates were incubated at 37°C for ~6 hr.

Substratum Preparation
Glass coverslips were mounted over holes in the bottom of 50 mm polystyrene Petri dishes. A 0.1 mg/ml poly L-lysine solution was applied to the coverslip to provide an adhesive surface for the binding of other molecules of interest. The glass coverslips were then washed two times with PBS and allowed to dry. Purified CSPGs were applied using cellulose strips (Whatman filter paper) in a pattern onto the poly L-lysine background (Modification of Snow et al., 2002). Laminin (25 µg/ml) was then applied to the entire coverslip, and incubated at room temperature for 1 hr. The substratum was then washed to remove unbound laminin or CSPGs from the substratum. The result was a patterned substratum consisting of alternating stripes of adsorbed CSPGs and laminin. (Figure 2)

Growth Cone Parameter Analysis
Growth Cone Measurements
A Zeiss Axiovision image analysis system was used to record time-lapse videos of individual DRG neurons as they approached and contacted CSPG stripes. The videos were then reviewed frame by frame and used to record measurements of numerous outgrowth parameters, with particular emphasis on the behavior of the leading “exploratory” component of the DRG neurons, called the growth cone (GC) (Figure 3 and Table 1).
Table 1: Partial List of Growth Cone Parameters Measured

<table>
<thead>
<tr>
<th>GROWTH CONE MORPHOLOGY</th>
<th>GROWTH CONE BEHAVIOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Cone Area</td>
<td>Growth on PG Stripe</td>
</tr>
<tr>
<td>Growth Cone Length</td>
<td>Growth Rate</td>
</tr>
<tr>
<td>Growth Cone Width</td>
<td>Growth Cone Acceleration</td>
</tr>
<tr>
<td>Number of Filopodia</td>
<td>Growth Cone Retraction</td>
</tr>
<tr>
<td>Max Filopodial Length</td>
<td>Growth Cone Turning</td>
</tr>
<tr>
<td>Total Length of Filopodia</td>
<td>Growth Cone Branching</td>
</tr>
</tbody>
</table>

Each of these GC parameters was measured in four different zones of neuronal outgrowth relative to the PG-adsorbed stripe. Note that Zone 1 neuronal responses were taken as the baseline values, as no contact with the experimental CSPG is made throughout this zone. (Figure 4)

Inhibitory Quotient (IQ) System Quantitative Assessment of Inhibition

The IQ system is a novel method by which to translate measurements of growth cone responses to Designer PGs into a quantitative, composite IQ score for each PG, to reflect the degree of inhibitory strength of each PG tested. To set the criteria, two PG standards were used that represent the extremes of growth cone behavior in response to this class of molecule: 1) bovine [adult] articular cartilage aggrecan (Sigma #A1960), which is highly decorated with GAG chains and induces complete inhibition of growth cone elongation; and 2) recombinant aggrecan expressed in the cell line CHO-745, which is deficient in xylosyl transferase, thus the CSPG is devoid of GAG chains, and is completely non-inhibitory to growth cone elongation. Growth cone responses to these two molecules set the criteria for analysis of all

Table 2: IQ System Scoring Criteria

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>PARAMETER</th>
<th>ZONE 2 CRITERIA (X VALUES)</th>
<th>ZONE 3 CRITERIA (Y VALUES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate</td>
<td>X &lt; 1.17</td>
<td>X between 1.17 and 1.43</td>
<td>X &gt; 1.43</td>
</tr>
<tr>
<td></td>
<td>X between -1.71 and -1.39</td>
<td>X &gt; -1.39</td>
<td>Y &lt; .20</td>
</tr>
<tr>
<td>Filopodial Behavior</td>
<td>X &lt; .98</td>
<td>X between .98 and 1.19</td>
<td>X &gt; 1.19</td>
</tr>
<tr>
<td></td>
<td>X between 1.04 and 1.28</td>
<td>X &gt; 1.28</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>X &lt; .92</td>
<td>X between .92 and 1.12</td>
<td>X &gt; 1.12</td>
</tr>
<tr>
<td>Morphology</td>
<td>X &lt; .92</td>
<td>X between .92 and 1.12</td>
<td>X &gt; 1.12</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>X &lt; .96</td>
<td>X between .96 and 1.18</td>
<td>X &gt; 1.18</td>
</tr>
</tbody>
</table>

Note: two criteria are used to derive zone 2 scores, etc. for zone 3
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subsequent Designer PGs, e.g., COS-7-derived aggrecan (see Results).

Development of the criteria (Table 2) for growth cone analysis required calculation of a single X and Y value for each of the two control PGs for every parameter. X and Y values summarize all experiments for a given PG (n = 3) and represent changes in behavior compared to growth on laminin alone (zone 1):

Comparison of behaviors in Zone 2 to Zone 1:
[ Avg. Z2/Z1 = X ]  (n=3)

Comparison of behaviors in Zone 3 to Zone 1:
[ Avg. Z3/Z1 = Y ]  (n=3)

Because the inhibitory trends for a given parameter were reflected in the X and Y values for each standard PG, a quantifiable basis for criteria development was established. Individual criteria were developed separately for zone 2 and zone 3 trends for each parameter and a point value of 0-2 was given depending on the extent to which the X and Y values reflected inhibitory responses (0 = least inhibitory, 2 = most inhibitory). The criteria for each parameter are integrated into category scores and then combined into a comprehensive scoring criterion that yields a composite IQ score ranging from 0-30 (least to most inhibitory). Although the raw data become compressed, the utility of the scoring criteria and computation of X and Y values is that they can be used to assign a composite IQ score to any experimental CSPG. Further, raw data is still taken into consideration, as is qualitative data for each growth cone’s response.

Results:
Growth Cone Behaviors Reflect the Degree of CSPG-Induced Inhibition

(Figure 5A) For the growth cone width parameter, both the X values (p = 0.027, t test) and the Y values (p = 0.011, t test) are significantly different for the bovine aggrecan positive control and the CHO 745 negative control. Such differences were quantified and incorporated into the IQ scoring criteria. (Figure 5B) Y values for both the positive and negative CSPG controls reveal significant differences (p = 0.039, t test) in growth cone area.

Composite IQ Scores Differ Between Designer PGs

The IQ system is sensitive in distinguishing the inhibitory capacity of three CSPGs with very different properties. The “COS-7 Designer PG” is a mutant form of aggrecan (see Methods). Note that its inhibitory properties are somewhat intermediate between those of bovine aggrecan and CHO-745 CSPGs. (Figure 6)
**Discussion:**

Two important conclusions immediately follow from this work. First, select behavioral and morphological parameters of DRG neurons are reliable predictors of CSPG-induced inhibition. This finding was readily shown by the statistically significant differences in responses of neurons to the bovine aggrecan and CHO-745 CSPG standards for the parameters, “growth cone area” and “growth cone width.” Significant differences were also observed for a number of other growth cone parameters, reinforcing the notion that such parameters reflect the inhibitory capacity of CSPGs.

Second, the differences we observed for growth cone responses to various CSPGs can be quantified and incorporated into a comprehensive scoring criterion. Because the two CSPG standards together comprise the entire spectrum of inhibitory responses (complete inhibition to no inhibition), values calculated for the bovine aggrecan established the criteria for maximum inhibition and values for CHO-745 CSPG established criteria for minimum inhibition. Values intermediate to those observed for the two standard CSPGs were assigned scores indicating intermediate inhibitory effects. Extension of this analysis to several other growth cone parameters then facilitates calculation of category scores, which are then combined to ultimately give the composite IQ score. The IQ system thus allows for the evaluation of any experimental CSPG (“Designer PG”).

A third result of this study thus far that is quite intriguing and novel in the field is that a blinded statistical analysis of the linear data for growth cone behaviors in response to CPSGs showed that values related to growth cone morphology significantly changed in the frame just following single filopodial contact with the inhibitor. For many years, researchers have questioned whether there is a cell surface receptor for CSPGs, with no resulting data to date to support this theory. That a cell can change its morphology immediately following contact with a single filopodium suggests that a receptor-mediated event is not out of the question, and supports further research in this regard. However, an alternative explanation might be effects of charge density, given the large degree of sulfation of CPSGs, or regulation of calcium-mediated events (Snow et al., 1992). These possibilities remain to be tested.

The IQ system analysis is not only consistent among different PG samples, but is also consistent among different users. This is true with respect to both growth cone measurement analyses as well as composite IQ score formulation. As such, it can be applied repeatedly to derive IQ scores for different CSPGs, and can be adopted for use on a more universal scale to test a wide variety of molecular cues. One NIH reviewer of this project stated the methodology “may become the standard in the field.” The results thus far support this prediction.

Further application of the IQ system of scoring with several Designer PG constructs will uncover the structural basis of CSPG-based outgrowth inhibition. The importance of this work is underscored by the fact that identification of these inhibitory domains should ultimately permit clinical applications to reduce inhibition, promote axonal regeneration, and counteract the utterly debilitating and presently irreversible nature of spinal cord injuries, to facilitate functional recovery.

**Acknowledgements**

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**Works Cited**