

## CS-4,6 is differentially upregulated in glial scar and is a potent inhibitor of neurite extension

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The precise contribution of different CS-GAGs to CSPG-mediated inhibition of axonal growth after CNS injury is unknown. Quantification of the CS-GAGs in uninjured and injured brain (scar tissue) using fluorophore-assisted carbohydrate electrophoresis (FACE) demonstrated that the dominant CS-GAG in the uninjured brain is CS-4 whereas, in glial scar, CS-2, CS-6, and CS-4,6 were over-expressed. To determine if the pattern of sulfation influenced neurite extension, we compared the effects of CS-GAGs with dominant CS-4, CS-6, or CS-4,6 sulfation to intact CSPG (aggrecan), chondroitin (CS-0), and hyaluronan on chick DRG neurite outgrowth. We report that CS-4,6 GAG, one of the upregulated CS-GAGs in astroglial scar, is potently inhibitory and is comparable to intact aggrecan, a CSPG with known inhibitory properties. Thus, a specific CS-GAG that is differentially over-expressed in astroglial scar is a potent inhibitor of neurite extension. These results may influence the design of more specific strategies to enhance CNS regeneration after injury.

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### Introduction

Chondroitin sulfate proteoglycans (CSPGs) are upregulated after injury in the central nervous system (CNS) (Asher et al., 2000, 2002; Fitch and Silver, 1997; Jones et al., 2002; Lemons et al., 1999; McKeon et al., 1995; Stichel et al., 1995; Tang et al., 2003) and define barriers for neurons in such structures as

perineuronal nets (Matthews et al., 2002), within the glial roof plate of the spinal cord and optic tectum (Snow et al., 1990a), hippocampus (Okamoto et al., 2001; Wilson and Snow, 2000), and the thalamocortical pathway (Fukuda et al., 1997). CSPGs consist of a protein core to which repeating disaccharides of glucuronic acid and galactosamine, termed glycosaminoglycans (CS-GAGs), are covalently coupled. While it is well established that CSPGs inhibit neurite outgrowth in vitro (Hynds and Snow, 1999; Johnson et al., 2002; Snow et al., 2001) and inhibit regeneration in vivo (Selles-Navarro et al., 2001; Zhang et al., 2001), the mechanism behind this inhibition remains to be elucidated, especially the relative contribution of the CSPG core protein vs. CS-GAGs. For example, the CSPGs NG2 (Dou and Levine, 1994) and versican V2 (Schmalfeldt et al., 2000) inhibit neurite extension via their protein cores, while brevican and neurocan require the presence of CS-GAGs for it to be inhibitory to neuronal extension (Yamada et al., 1997) or for neuronal attachment (Talts et al., 2000), respectively.

While CS-GAGs have a significant structural diversity (Fig. 1), the nature and profile of CS-GAGs in the astroglial scar have only recently been investigated (Properzi et al., 2005). The contribution of CS-GAGs to regenerative outcomes is significant as recent studies have demonstrated that digestion of CS-GAGs improves axonal regeneration after spinal cord injury (Bradbury et al., 2002; Moon et al., 2001). Additionally, CS-GAGs are concentrated at the tectal midline in the developing hamster, which serves as a putative barrier to retinal axon growth (Hoffman-Kim et al., 1998). However, direct in vitro evidence for the contribution of CS-GAGs to neurite extension has been inconclusive. For instance, while some CS-GAGs were inhibitory (Becker and Becker, 2002; Brittis and Silver, 1994; Meiners et al., 1995), other CS-GAGs did not display the same inhibitory character (Challacombe and Elam, 1997; Clement et al., 1999; Faissner et al., 1994; Feraud-Espinosa

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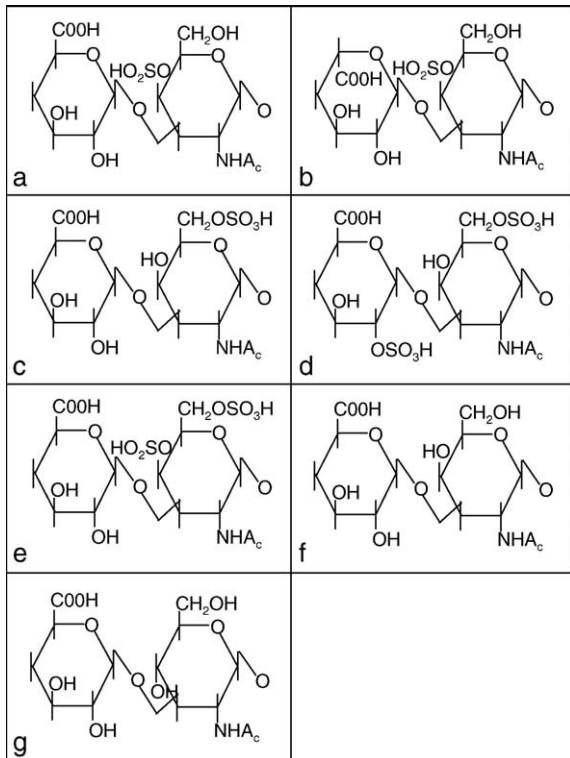


Fig. 1. Schematic structure of various chondroitin sulfate glycosaminoglycans and hyaluronan: (a) chondroitin-4-sulfate consists of 0S glucuronic acid and 4S galactosamine ( $\Delta$ glcA- $\beta$ 1,3-4S-galNAc). Commercially, CS-A contains a high amount of chondroitin-4-sulfate. (b) Dermatan-4-sulfate consists of 0S iduronic acid and 4S galactosamine ( $\Delta$ iduA- $\alpha$ 1,3-4S-galNAc). Commercially, CS-B contains a high amount of dermatan-4-sulfate. (c) Chondroitin-6-sulfate consists of 0S glucuronic acid and 6S galactosamine ( $\Delta$ glcA- $\beta$ 1,3-6S-galNAc). Commercially, CS-C contains a high amount of chondroitin-6-sulfate. (d) Chondroitin-2,6-sulfate consists of 2S glucuronic acid and 6S galactosamine ( $\Delta$ 2S-glcA- $\beta$ 1,3-6S-galNAc). Commercially, CS-D contains a high amount of chondroitin-2,6-sulfate. (e) Chondroitin-4,6-sulfate consists of 2S glucuronic acid and 6S galactosamine ( $\Delta$ glcA- $\beta$ 1,3-4,6S-galNAc). Commercially, CS-E contains a high amount of chondroitin-4,6-sulfate. (f) Chondroitin-0-sulfate consists of 0S glucuronic acid and 0S galactosamine. Commercially, chondroitin contains high amount of chondroitin-0-sulfate ( $\Delta$ glcA- $\beta$ 1,3-galNAc). (g) Hyaluronan consists of 0S glucuronic acid and 0S glucosamine ( $\Delta$ glcA- $\beta$ 1,3-4,6S-GlcNAc). Ac represents an acetate group.

et al., 1994; Nadanaka et al., 1998). In these studies, no clear distinction between soluble CS-GAGs and immobilized CS-GAGs was made, and these may have differential effects (Snow et al., 1996). As CS-GAGs are generally immobilized in scar tissue, we designed a 3D culture system with CS-GAGs immobilized on agarose hydrogels for the study of CS-GAGs' effects on E9 chick DRG neurite extension.

In this study, we demonstrate that the CS-GAG expression profile is quite distinct and different in scar tissue compared to normal uninjured cortex with CS-2, CS-6, and CS-4,6 GAGs being differentially upregulated in the glial scar. Furthermore, we demonstrate that when the same molar concentration of CS-GAGs are immobilized to our hydrogel backbone, different levels of inhibition of neurite extension occur *in vitro* based on the pattern of sulfation. While all the CS-GAGs upregulated in glial scar inhibit neurite extension significantly even in the absence of their protein cores, CS-4,6 is a particularly potent inhibitor of neurite extension.

## Results

*CS-GAG expression is significantly different in the scar tissue than that in normal cortex*

In the first set of filter samples, FACE revealed that chondroitin-4,6-sulfate (0S-glucuronic acid-4,6S-*N*-acetyl galactosamine), chondroitin-2-sulfate (2S-glucuronic acid-0S-*N*-acetyl galactosamine), and chondroitin-6-sulfate (0S-glucuronic acid-6S-*N*-acetyl galactosamine) were upregulated in the injured cortex (data not shown). A second set of filter samples were processed using chondro-6-sulfatase to quantitatively determine the true CS-GAG distribution independent of the AMAC contaminant. Our FACE analysis confirmed that the GAG profiles of normal cortex and scar tissue are significantly different. Within the normal, uninjured cortex, most of the CS-GAG is chondroitin-4-sulfated (0S-glucuronic acid-4S-*N*-acetyl galactosamine) with some chondroitin (0S-glucuronic acid-0S-*N*-acetyl galactosamine) and chondroitin-6-sulfate also present (Fig. 2a, lane 4; Table 1). In contrast, there are four main types of sulfated GAG disaccharides within scar tissue, chondroitin-4,6-sulfate, chondroitin-6-sulfate, chondroitin-2-sulfate, and chondroitin-4-sulfate (Fig. 2b, lane 4; Table 1). The scar samples (Fig. 2b, lanes 3 and 4) also contained greater glucose content than the normal cortex samples (Fig. 2a, lanes 3 and 4).

Controls were run on the brain and injured cortex samples in which no enzyme was added. In the no enzyme control lanes (Fig. 2a, lane 2; Fig. 2b, lane 5), the presence of bands in the  $\Delta$ Di6S region and the regions between the  $\Delta$ Di6S and 4S-acetyl galactosamine (4S GalNAc) are due to the presence of AMAC contaminant (Calabro et al., 2000b). Monosaccharide and disaccharide standards (Fig. 2a, lanes 1 and 5; Fig. 2b, lane 1) were run to identify monosaccharides and disaccharides in the sample lanes.

To verify the CS-GAG profile in the injured cortex, and to determine whether the identified disaccharides in the injured brain similarly shifted following sulfatase treatment, filters were removed after 30 days of implantation and subjected to FACE. When the injured cortex samples were run without enzyme digestion, the prominent band resided in the  $\Delta$ Di6S region (Fig. 2b, lane 5) coexistent with the AMAC contaminant. When the injured cortex samples were treated with enzyme cocktail, the presence of  $\Delta$ Di4,6S and  $\Delta$ Di4S bands and a slight  $\Delta$ Di2S band was apparent (Fig. 2b, lane 4). Upon treatment with chondro-6-sulfatase, the  $\Delta$ Di4,6S band disappears and the  $\Delta$ Di4S band becomes more intense (Fig. 2b, lane 3). In addition, the chondro-6-sulfatase digested the chondroitin-6-sulfate present in the  $\Delta$ Di6S band since there is strong  $\Delta$ Di0S band now present in the injured cortex treated with chondro-6-sulfatase lane (Fig. 2b, lane 3) that was not present in the injured cortex sample that did not undergo chondro-6-sulfatase treatment (Fig. 2b, lane 4). Maltose standard (Fig. 2b, lane 2) and the glucose standard within the disaccharide and monosaccharide and standard lane (Fig. 2b, lane 1) was used to quantify the amount of CS-GAG in the injured cortex lanes.

To determine the efficiency of chondro-6-sulfatase's ability to convert chondroitin-4,6-sulfate to chondroitin-4-sulfate, we measured the intensity change in the chondroitin-4,6-sulfate following chondro-6-sulfatase treatment (Fig. 2b). From the intensity readings, chondro-6-sulfatase converted 95% of chondroitin-4,6-sulfate to chondroitin-4-sulfate. To calculate the amount of  $\Delta$ Di6S in the filter samples independent of the AMAC contaminant, it was

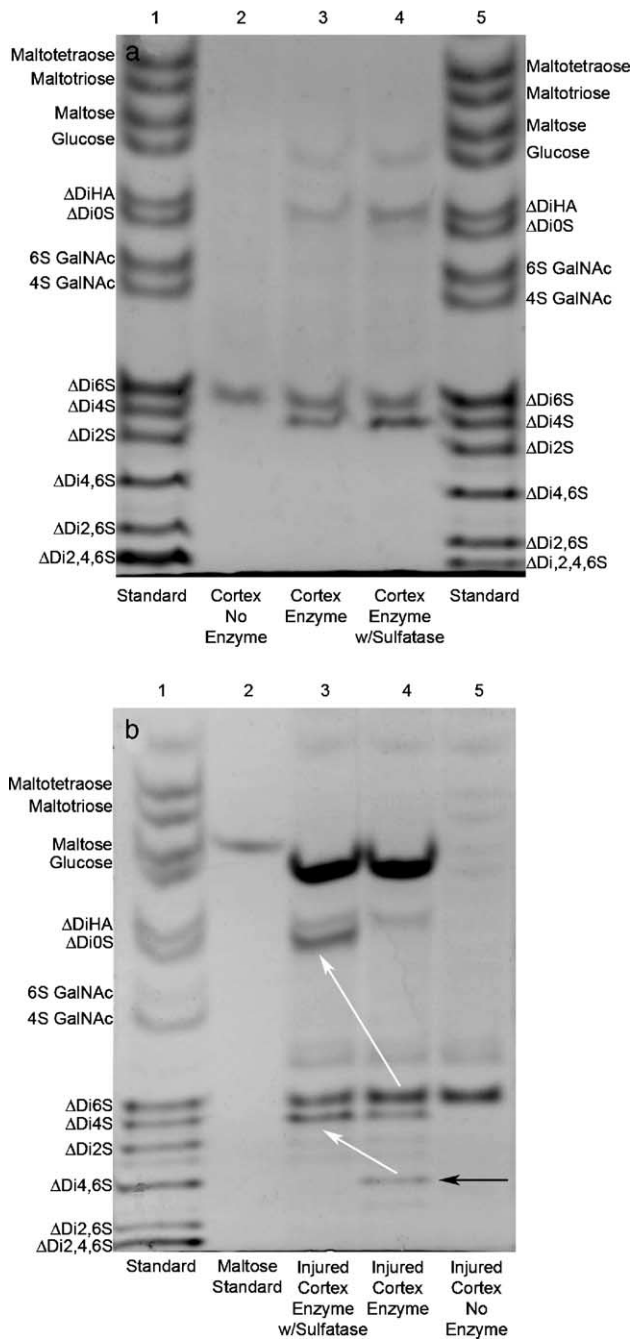


Fig. 2. FACE analysis of normal and injured rat cortex. (a) Normal cortex: lane 1: GAG and sugar standards; lane 2: cortex without enzyme treatment; lane 3: cortex with enzyme treatment; lane 4: cortex with enzyme and chondro-6-sulfatase treatment; lane 5: GAG and sugar standards. (b) Injured cortex; lane 1: GAG and sugar standards; lane 2: maltose standard; lane 3: injured cortex with enzyme and chondro-6-sulfatase treatment; lane 4: injured cortex with enzyme treatment; lane 5: injured cortex without enzyme treatment. The black arrow (b) shows the prominent  $\Delta$ Di4,6S present in the injured cortex sample. The white arrow indicates the removal of chondroitin-4,6-sulfate within the injured cortex with enzyme lane (b, lane 4) to the  $\Delta$ Di4S band within the injured cortex with enzyme and chondro-6-sulfatase lane (b, lane 3). The second white arrow (b) shows the removal of chondroitin-6-sulfate within the injured cortex with enzyme lane (b, lane 4) to the  $\Delta$ Di0S band within the injured cortex with enzyme and chondro-6-sulfatase lane (b, lane 3).

Table 1  
GAG analysis of cortex and injured cortex

	Disaccharide	Percent of total GAG
Cortex	0S	5
	4S	91
	6S	4
Injured cortex	2S	8
	4S	25
	6S	50
	4,6S	17

FACE analysis of cortex and injured cortex within the rat. The amount of GAG disaccharide was summed in either cortex or injured cortex, and the percentage of a particular sulfated disaccharide was determined.

determined from the intensity readings that the chondro-6-sulfatase would convert around 95% of the  $\Delta$ Di6S to  $\Delta$ Di0S. Therefore, the amount of  $\Delta$ Di6S in the filter sample was computed, eliminating the need of subtracting away the AMAC contaminant band. Using this value for the amount of chondroitin-6-sulfate in the  $\Delta$ Di6S band, the distribution of sulfated GAG was determined; with the most prominent CS-GAG being the chondroitin-6-sulfate (50%), followed by chondroitin-4-sulfate (25%), chondroitin-4,6-sulfate (17%), and chondroitin-2-sulfate (8%). In the normal adult cortex, most of the CS-GAG was 4-sulfated (91%) with some chondroitin-6-sulfate (4%) and chondroitin (5%) (Table 1).

To ensure that the blank filters did not contain any sulfated CS-GAGs, blank, non-implanted filters were examined using FACE. When the blank filter samples were treated with or without the enzyme, both lanes had a band that ran similarly to  $\Delta$ Di6S, indicating the presence of the AMAC contaminant (Fig. 3a, lanes 1 and 2). If the blank filter sample had sulfated GAG disaccharides, then the enzyme-treated blank filter sample would have bands within the sulfated GAG disaccharide region of the FACE gel. Since a band is present only at the  $\Delta$ Di6S position and the intensity of this band is not different than the  $\Delta$ Di6S band in the non-enzyme-treated blank filter sample, the signal is likely the result of AMAC contaminant and not the presence of  $\Delta$ Di6S. Thus, these results demonstrate that the blank filter samples do not contain any sulfated CS-GAG disaccharides.

If the amount of  $\Delta$ Di6S was to be quantified following chondro-6-sulfatase treatment, it was necessary to determine if the AMAC contaminant was affected by chondro-6-sulfatase treatment. The AMAC contaminant band (Fig. 3b, lane 2) when treated with chondro-6-sulfatase (Fig. 3b, lane 3) was not moved. Thus, when a sample was treated with chondro-6-sulfatase, the resulting  $\Delta$ Di0S band is from the  $\Delta$ Di6S within a sample, and not from the contaminant. The AMAC contaminant band is located in the  $\Delta$ Di6S region as verified when comparing the contaminant band to monosaccharide and disaccharide standards (Fig. 3b, lane 1).

To determine whether the band seen in Fig. 2b was  $\Delta$ Di4,6S, three standards were subjected to chondro-6-sulfatase treatment (Fig. 3c) to determine if the enzyme would move the standards (in particular the  $\Delta$ Di4,6S band to the  $\Delta$ Di4S position) to their corresponding lanes in a quantitative manner. The  $\Delta$ Di6S standard (Fig. 3c, lane 2) when treated with chondro-6-sulfatase converted the  $\Delta$ Di6S standard to the  $\Delta$ Di0S position within the FACE gel (Fig. 3c, lane 3). The  $\Delta$ Di2,4,6S standard (Fig. 3c, lane 5) when treated with chondro-6-sulfatase converted the  $\Delta$ Di2,4,6S standard to the  $\Delta$ Di2,4S position within the FACE gel (Fig. 3c, lane 4). The  $\Delta$ Di4,6S standard (Fig. 3c, lane 7) when treated with chondro-6-

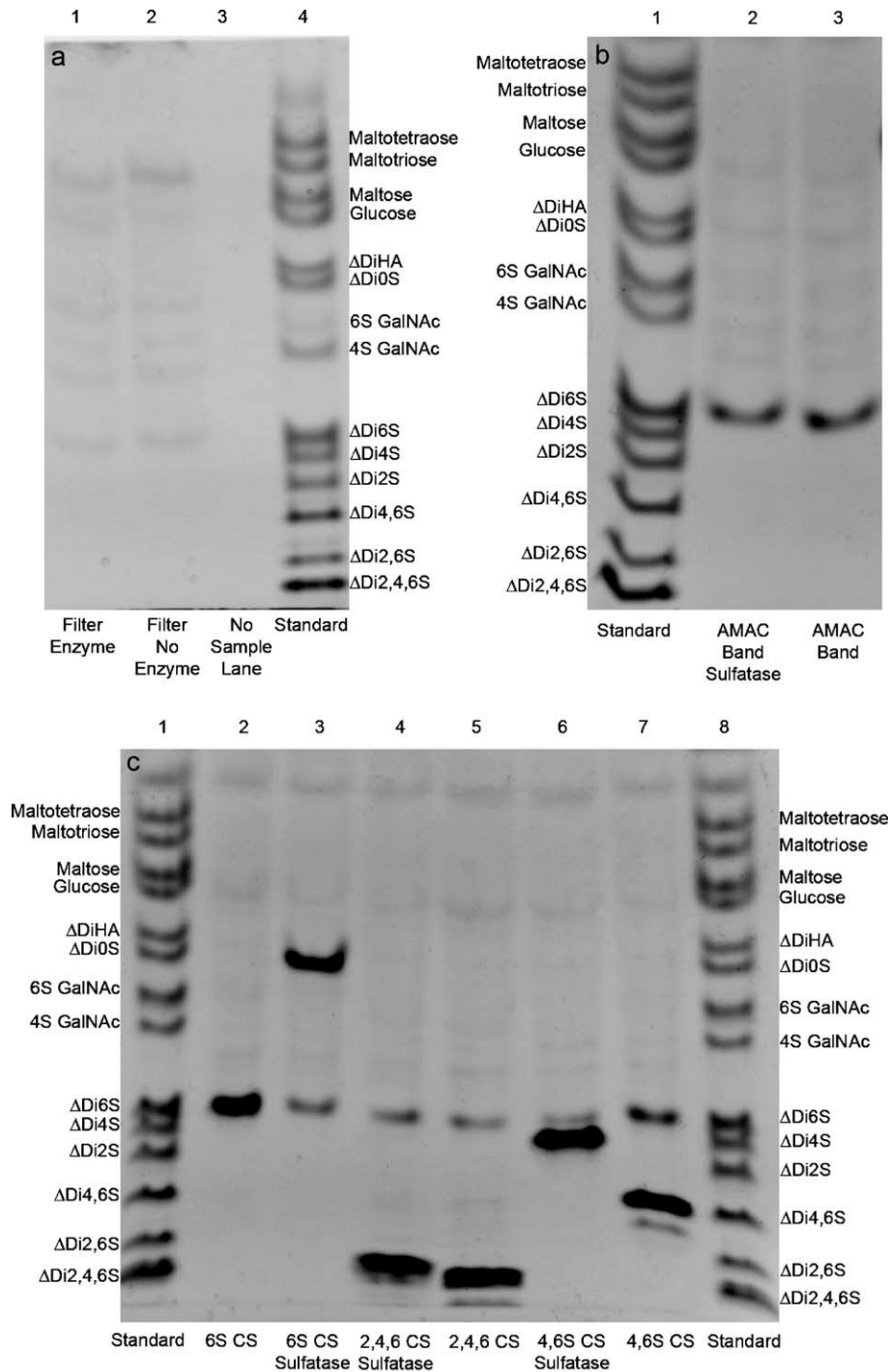


Fig. 3. FACE results of blank filter samples, AMAC contaminant treated with and without chondro-6-sulfatase, and chondro-6-sulfatase-treated standards. (a) Blank filter samples: lane 1: blank filter with enzyme treatment; lane 2: blank filter without enzyme treatment; lane 3: blank lane; lane 4: GAG and sugar standards. (b) AMAC contaminant treatment with chondro-6-sulfatase: lane 1: GAG and sugar standards; lane 2: blank sample with AMAC contaminant treated with chondro-6-sulfatase; lane 3: blank sample with AMAC contaminant. (c) CS-GAG standard treatment with chondro-6-sulfatase; lane 1: GAG and sugar standards; lane 2: 6S CS ( $\Delta$ Di6S) standard; lane 3: 6S CS standard treated with chondro-6-sulfatase; lane 4: 2,4,6S CS ( $\Delta$ Di2,4,6S) standard treated with chondro-6-sulfatase; lane 5: 2,4,6S CS standard; lane 6: 4,6S CS ( $\Delta$ Di4,6S) standard treated with chondro-6-sulfatase; lane 7: 4,6S CS standard; lane 8: GAG and sugar standards.

sulfatase converted the  $\Delta$ Di4,6S standard to the  $\Delta$ Di4S level (Fig. 3c, lane 6). The  $\Delta$ Di4,6S standard ran in a doublet manner but both bands were removed following chondro-6-sulfatase treatment. Monosaccharide and disaccharide standards (Fig. 3c, lanes 1 and

8) were again run to identify monosaccharide and disaccharides in the sample lanes. Chondroitin-6-sulfatase converted approximately 95% of the standards to their post-sulfatase-treated bands within the FACE gel (data not shown).

### Confirmation that the chondroitin-4,6-sulfate band is chondroitin-4,6-sulfate

To verify that the CS-GAG seen in the  $\Delta$ Di4,6S band within the enzyme-treated filter samples was chondroitin-4,6-sulfate, chondroitin-4,6-sulfate standard was subjected to the FACE analysis. This standard (Fig. 4, lane 2) shows excellent alignment with the chondroitin-4,6-sulfate standard within the monosaccharide and disaccharide CS-GAG standard lane (Fig. 4, lane 1). When the injured cortex was enzymatically treated, the resulting  $\Delta$ Di4,6S band (Fig. 4, lane 3) aligns well with chondroitin-4,6-sulfate standard (Fig. 4, lane 2). When the enzyme-treated injured cortex sample is mixed with the chondroitin-4,6-sulfate standard, the  $\Delta$ Di4,6S band is more intense and the two bands do not run into two distinct regions of the FACE gel (Fig. 4, lane 4); verifying that the band seen in the injured cortex sample is chondroitin-4,6-sulfate.

### DRG neurite extension is significantly inhibited by immobilized, sulfated GAGs/PG

To determine if the different CS-GAGs identified in gliotic tissue could have different degrees of inhibitory potential, DRGs were cultured in different CS-GAG containing agarose gel cultures. Also to determine if the presence of sulfate groups were necessary for inhibition, control GAGs without any sulfate, chondroitin and hyaluronan were also immobilized to agarose gels. To determine the potency of inhibition, gels with equimolar amounts of CS-GAG/volume of agarose gel were fabricated. We initially used

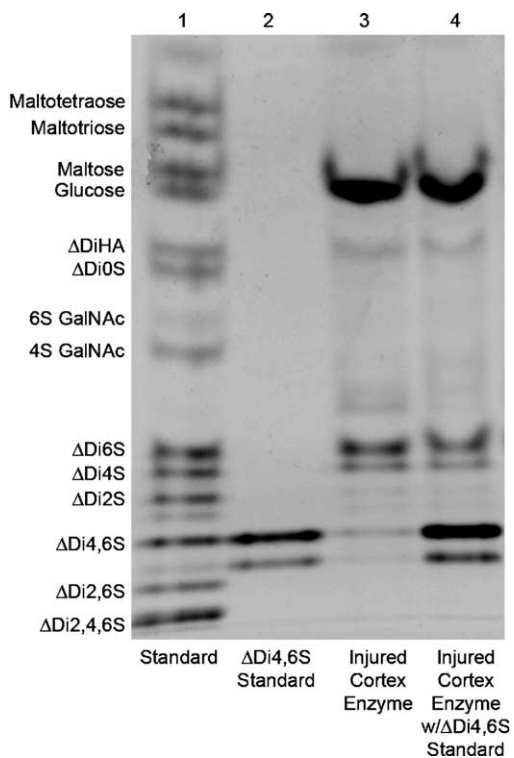


Fig. 4. FACE results of 4,6S CS standard, injured cortex treated with enzyme, and 4,6S CS standard mixed in with injured cortex treated with enzyme; lane 1: GAG and sugar standards; lane 2: 4,6S CS standard; lane 3: injured cortex with enzyme; lane 4: injured cortex with enzyme treatment mixed with 4,6S CS standard.

FACE to characterize the sulfate profile of different GAGs. Commercially available CS-B and bovine nasal septum aggrecan both have significant amounts of 4-sulfated GAG. The aggrecan sample also has 6-sulfated and 0-sulfated GAGs, while the CS-B sample has some 6-sulfated, 2,6-sulfated GAG, and 0-sulfated GAG (Table 2). DRGs grown in the presence of immobilized, sulfated CS-GAGs or intact aggrecan had significantly shorter neurites than DRGs grown in agarose. DRGs in the presence of just agarose had long neurites after 48 h (Fig. 5a). When DRGs were grown in the presence of CS-B (Fig. 5b), neurites still emanated into the surrounding environment, but there were generally fewer and shorter neurites radiating from the explant. Those DRGs grown in the presence of CS-C had neurites similar in length to those in agarose but had fewer total processes (Fig. 5c). DRGs introduced to gels with either CS-E (Fig. 5d) or aggrecan (Fig. 5e) had very little growth to no growth.

Two methods were used to quantitatively characterize neurite outgrowth in the various gels. To determine the maximum neurite extension in each gel, the length of the eight longest neurites for each DRG was quantified. In this case, each of the sulfated CS-GAG/PG gels inhibited neurite outgrowth when compared to agarose gels as determined by a general linear ANOVA model ( $P < 0.05$ ). When using pairwise comparisons (Tukey 95% simultaneous confidence intervals), all pairs were statistically significant except when comparing CS-E to aggrecan. When the average neurite lengths were analyzed (50 data point analysis), all of CS-GAG/PG gels were inhibitory statistically, except when comparing CS-E gel to aggrecan gel and agarose gel to CS-C gel. After 48 h of culture time, the average neurite length (eight longest neurites) from the four DRGs was as follows: agarose— $1090 \pm 96 \mu\text{m}$ , CS-C— $909 \pm 126 \mu\text{m}$ , CS-B— $633 \pm 128 \mu\text{m}$ , CS-E— $31 \pm 57 \mu\text{m}$ , and aggrecan— $38 \pm 70 \mu\text{m}$  (Fig. 6a). The average neurite length from the four DRGs was as follows: agarose— $834 \pm 142 \mu\text{m}$ , CS-C— $841 \pm 154 \mu\text{m}$ , CS-B— $552 \pm 131 \mu\text{m}$ , CS-E— $22 \pm 40 \mu\text{m}$ , and aggrecan— $24 \pm 44 \mu\text{m}$  (Fig. 6b).

Quantitatively, gels with chondroitin (having the same basic structure as the other sulfated CS-GAGs) had shorter neurites (Fig. 7b) than neurites in agarose (Fig. 7a). When neurites were grown in the presence of hyaluronan (having a different disaccharide structure than chondroitin), neurite length (Fig. 7c) was more comparable to agarose. Using a general linear ANOVA analysis, the mean values for the different gel conditions in the eight longest neurite analysis were different statistically ( $P < 0.05$ ). In the Tukey pairwise analysis, all pairs differed statistically. When conducting the general linear ANOVA analysis and the Tukey pairwise analysis for the average neurite length (50-point analysis), the means and the pairwise analysis all differed statistically ( $P < 0.05$ ). After 48 h of culture time, the average neurite length from the three DRGs that had the longest neurites was as follows: agarose— $1106 \pm 101 \mu\text{m}$ , hyaluronan— $978 \pm 169 \mu\text{m}$ , and chondroitin— $707 \pm 107 \mu\text{m}$  (Fig. 8a). The average neurite length (50 points of analysis) from the three DRGs was as follows: agarose— $853 \pm 150 \mu\text{m}$ , hyaluronan— $748 \pm 194 \mu\text{m}$ , and chondroitin— $682 \pm 106 \mu\text{m}$  (Fig. 8b).

### Comparing GAG concentration in cortex to that in our culture system

The amount of sulfated GAG in normal, uninjured cortex is  $1.08 \mu\text{g}$  of sulfated GAG per mg of cortex (CS-B calibration) or  $0.97 \mu\text{g}$  of sulfated GAG per mg of cortex (CS-C calibration) (wet weight).

Table 2  
GAG distribution for various sources of GAG/PG

Disaccharide type	0S (%)	6S (%)	4S (%)	2,6S (%)	4,6S (%)
Squid cartilage (CS-E) <sup>a</sup>	5.9	9.6	22.9	–	61.5
Shark cartilage (CS-C) <sup>a</sup>	1.7	72.9	15.4	9.3	0.6
Porcine intestinal mucosa (CS-B) <sup>b</sup>	4.6	9.3	81.4	4.7	–
Aggrecan <sup>b</sup>	12.7	25	62.3	–	–

Sulfation patterns of resolved disaccharides from commercial sources of GAG and a private source of proteoglycan. GAG distribution data from squid cartilage (CS-E) and shark cartilage (CS-C) was obtained from previously published data (Yoshida et al., 1989). GAG distribution data from porcine intestinal mucosa (CS-B) and aggrecan was derived using FACE data.

<sup>a</sup> Information obtained from Yoshida et al.

<sup>b</sup> Information obtained from FACE analysis.

Thus, the concentration of sulfated GAG within cortex is approximately 1.0 µg/mg. Our gel samples were standardized to contain a sulfated GAG concentration of 0.5 mg of sulfated GAG per milliliter gel. Each milliliter of gel has a mass of approximately 1000 mg. Using this conversion to relate the amount of sulfated GAG in cortex

to that within our gels, our gels have a sulfated GAG concentration of 0.5 µg of sulfated GAG per mg of gel (wet weight), or about half the amount present per mg in the brain.

## Discussion

It is now generally accepted that CSPGs are among a number of axon growth inhibitory molecules expressed in the adult CNS. Recent studies focusing on alleviation of CSPG inhibition demonstrate that in vivo delivery of the CS-GAG degrading enzyme chondroitinase ABC results in enhanced regeneration (Bradbury et al., 2002; Moon et al., 2001). While this suggests a role for CS-GAGs, little is known regarding the contribution of specific CS-GAGs to inhibition in the CNS. A diverse group of CS-GAGs are expressed in the CNS including CS-2 (2S D-glucuronic acid-0S D-N-acetylgalactosamine), CS-4 (0S D-glucuronic acid, 4S D-N-acetylgalactosamine), CS-6 (0S D-glucuronic acid, 6S D-N-acetylgalactosamine), CS-2,6 (2S D-glucuronic acid, 6S D-N-acetylgalactosamine), and CS-4,6 (0S D-glucuronic acid, 4,6S D-N-acetylgalactosamine). This rich diversity is generated by differential expression of a family of

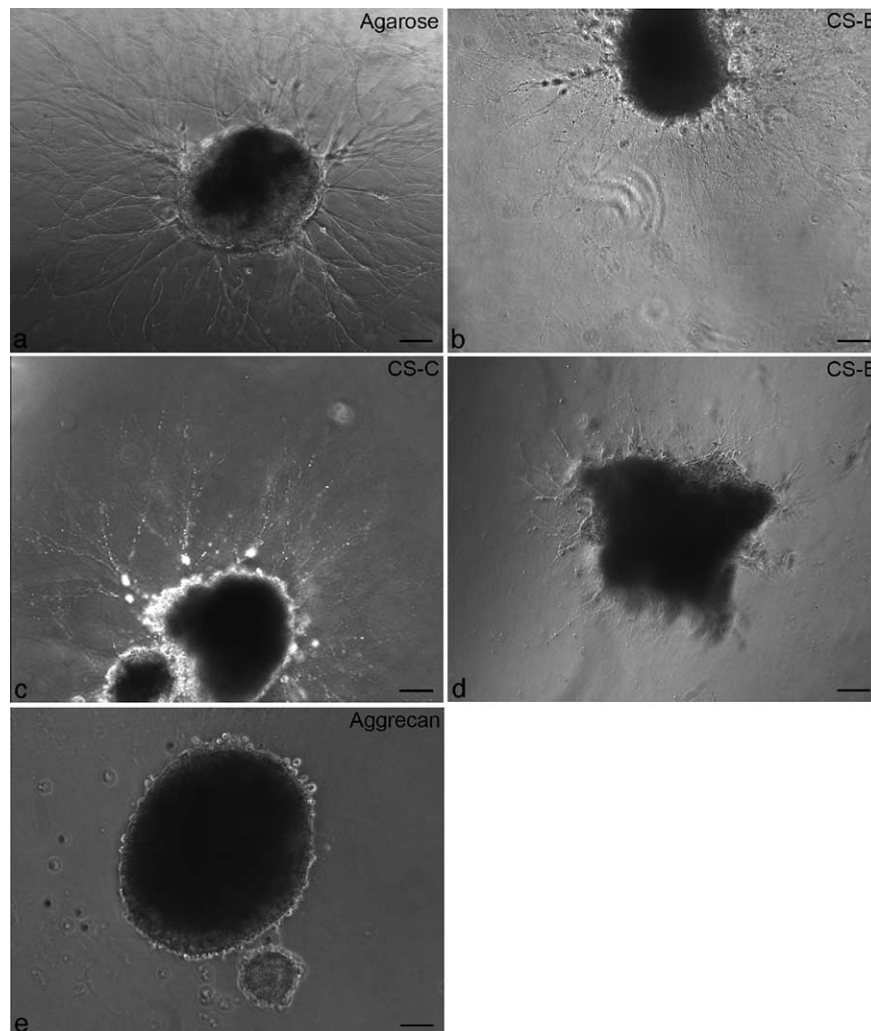


Fig. 5. DRG neurons grown in agarose and various agarose modified gels after 48 h of culture time. (a) DRGs grown in unmodified agarose; (b) DRGs grown in CS-B agarose; (c) DRGs grown in CS-C agarose; (d) DRGs grown in CS-E agarose; (e) DRGs grown in CSPG aggrecan agarose. Scale bar = 100 µm.

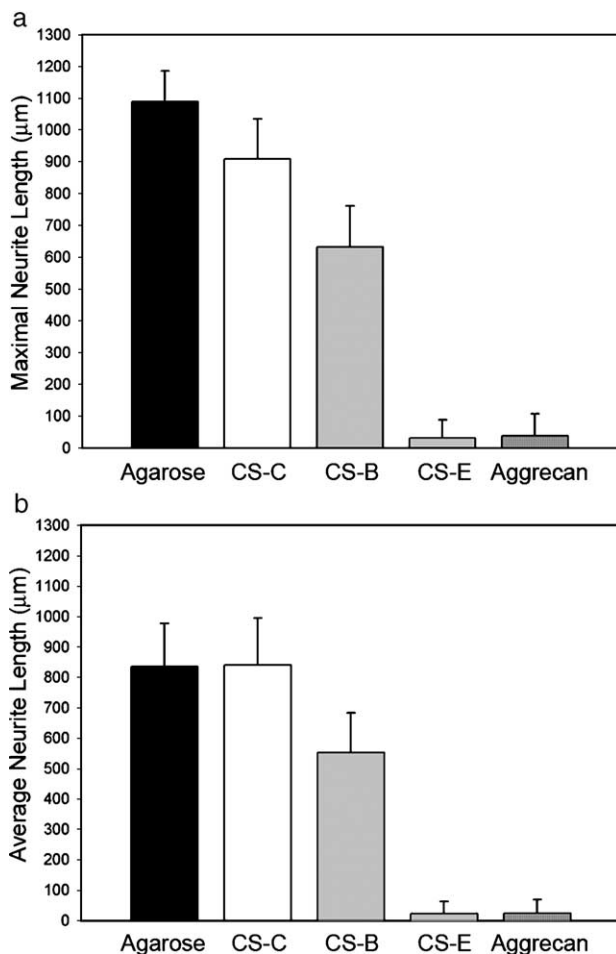


Fig. 6. Neurite outgrowth from E9 stage chick DRGs in various sulfated GAG/PG agarose hydrogel systems. (a) The eight longest neurites from each DRG were averaged and compared to other DRGs in each gel condition. Using a general linear ANOVA model, means for all of the gel conditions were statistically different ( $P < 0.05$ ). Using a Tukey 95% simultaneous confidence interval for pairwise comparisons, all pairwise comparisons were statistically significant ( $P < 0.05$ , except when comparing CS-E to aggrecan). (b) The average neurite length for each DRG was determined using 50 lines from the explant to a line that connected the distinguishable growth cones of the neurites. Using a general linear ANOVA model, means for all of the gel conditions were statistically different ( $P < 0.05$ ). Using Tukey 96% simultaneous confidence interval for pairwise comparisons, all pairwise comparisons were statistically significant ( $P < 0.05$ ), except when comparing agarose to CS-C and CS-E to aggrecan.

enzymes, sulfotransferases (Properzi et al., 2003). A recent study suggests that the pattern of CS-GAG sulfation changes after cortical stab wound injury, where there is an increase in percentage of chondroitin-6-sulfate, even though the most predominant sulfated GAG in the injured cortex samples is still 4-sulfated (Properzi et al., 2005). Similar to our study, CS-GAGs associated with nitrocellulose filters were also analyzed 7 days after implantation and again the most prominent CS-GAG is the 4-sulfated type, but with upregulated  $\Delta$ Di0S,  $\Delta$ Di6S, and  $\Delta$ Di4,6S (Properzi et al., 2005). In contrast, our filters were harvested 30 days after injury to make certain that the CS-GAG harvested was from mature scars. It is possible that the time the filter resides in the brain is an important variable in determining

the CS-GAG distribution in scar, since our CS-GAG distribution from the filter samples is quite different. Additionally, a number of recent reports demonstrate the increased expression of different CSPG core proteins (Dobbertin et al., 2003; Tang et al., 2003). While the underlying assumption may be that the pattern of sulfation on GAGs linked to these cores is similar to that seen in the intact cortex, another possibility is that the CS-GAG on these protein cores are differentially sulfated in the injured cortex.

We sought to determine if the profile of CS-GAG expression in injured cortex is different from uninjured cortex using FACE analysis of normal versus gliotic tissue harvested from nitrocellulose filters implanted into the adult rat cortex (McKeon et al., 1995, 1999). We initially examined alternative methods of making injuries such as contusion or stab wounds followed by laser-assisted dissection of GFAP stained tissue sections to isolate scar. However, it was very difficult to consistently obtain sufficient quantities of uncontaminated scar tissue using these methods (data not shown). We subsequently chose to use implanted nitrocellulose filters to generate scar tissue as this technique allows us to isolate scar tissue generated in vivo with little contamination from surrounding normal tissue. Therefore, the nitrocellulose filter implantation affords the most accurate method of isolating scar tissue. Nitrocellulose filters implanted into the CNS stimulate the formation of a glial scar that contains varying cell types including astrocytes, fibroblasts, macrophages, and some endothelial cells (Rudge and Silver, 1990). We implanted filters into the cortex of 45-day-old rats and harvested them 30 days later in order to allow for a thorough deposition of ECM molecules, including CSPGs, in a “mature” glial scar. The percentage of cell types in these scars is not expected to vary greatly from a published report (Rudge and Silver, 1990) demonstrating that of the cells associated with filters implanted into 20-day-old rats for 10 days, 65% were astrocytes, 16% were microglia/macrophages, 15% were fibroblasts, and 4% were endothelial cells. Additionally, the CSPGs neurocan and phosphacan (McKeon et al., 1999) are also present in the ECM associated with these filters, but the GAG profile in this tissue is not known. Biochemically, we used FACE analysis as it is capable of quantitatively distinguishing between the various CS-GAGs with different patterns of sulfation (Calabro et al., 2000a,b, 2001).

Our FACE data demonstrate that the CS-GAG expression profile in the normal, uninjured cortex is significantly different from the CS-GAG expression profile in scar tissue (Fig. 2). Of particular significance is our observation that a new CS-GAG, CS-4,6 is exclusively upregulated in scar tissue and not in uninjured adult cortex. As our subsequent data demonstrate (Figs. 5 and 6), this is a potentially inhibitory GAG with its inhibitory potential being comparable to intact aggrecan. It is reasonable to conclude that CS-4,6 is likely to contribute significantly to scar-mediated inhibition in vivo. Our data also demonstrate that the dominant CS-GAG in the uninjured cortex is CS-4, whereas the dominant CS-GAGs in the scar tissue is CS-6. These data are consistent with previous biochemical characterizations of specific CSPGs in the adult CNS, which are predominantly CS-4 (Flaccus et al., 1991). Likewise, previous immunocytochemical studies have demonstrated that after cortical injury, a CS-6 epitope is predominantly revealed following chondroitinase ABC digestion (McKeon et al., 1991). This result is also consistent with another report suggesting differential upregulation of specific sulfotransferases, enzymes responsible for the diversity of sulfation on CSPGs following injury (Properzi et al., 2005).

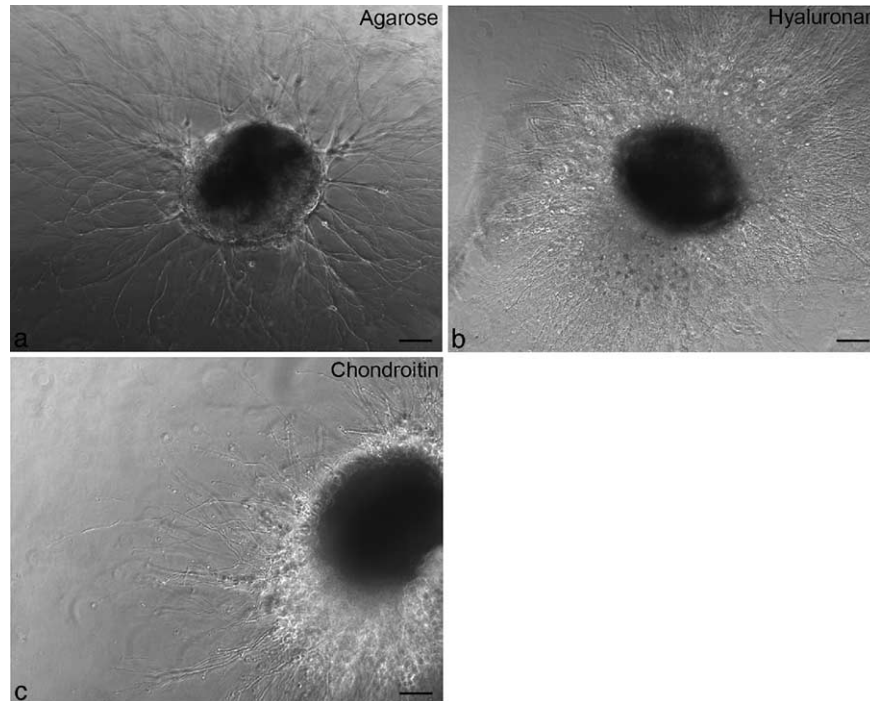


Fig. 7. (a) Those DRGs grown in plain agarose had neurons with significant sprouting into the surrounding gel. (b) DRGs grown in the presence of hyaluronan had similar neurite outgrowth to those neurites in agarose. (c) DRGs grown in the presence of chondroitin were significantly inhibited compared to gels with no CS-GAGs. Scale bar = 100  $\mu$ m.

Given our finding that CS-GAGs are differentially expressed in the injured brain and previous work demonstrating CS-GAG-mediated axon growth inhibition, we sought to determine whether the specific GAGs upregulated in scar tissue are *more* inhibitory than those expressed in uninjured cortex. Studies to date that directly attempt to analyze the effects that CS-GAGs with different patterns of sulfation have on growth cones are confusing and contradictory, although the chondroitinase ABC literature indirectly suggests a significant inhibitory role for CS-GAGs. For instance, when CS-D (CS-2,6) was adsorbed onto glass coverslips with poly-DL-ornithine, enhanced neurite outgrowth of embryonic day 18 hippocampal neurons (Nadanaka et al., 1998) was observed. The other double-sulfated glycosaminoglycan, CS-E (0-sulfated glucuronic acid with a 4,6-sulfated galactosamine), reportedly stimulated neurite outgrowth from embryonic hippocampal neurons, while single sulfated glycosaminoglycans, CS-A, CS-B, and CS-C were reported to be neither stimulatory nor inhibitory (Clement et al., 1999). However, we have previously reported that CS-B-bound agarose gels were inhibitory even without their protein cores (Dillon et al., 2000; Yu and Bellamkonda, 2001). Previous studies from our laboratory attempted to determine whether specific CS-GAGs affected neuronal outgrowth differently (Gilbert and Bellamkonda, 2003). However, in those preliminary studies, all DRGs within a hydrogel with a covalently coupled CS-GAG were analyzed to determine the eight longest neurites within a particular hydrogel system. In the current study, only those DRGs with neurites emanating in all directions (if such were available—in the case of aggrecan and CS-E there was always little if any neurite outgrowth in all DRGs examined) were analyzed to insure that the growth capacity of the DRGs analyzed was consistent among groups.

One possible reason for these conflicting results is that the typical preparation for in vitro study of CS-GAGs uses physical adsorption onto glass or tissue culture polystyrene as a method of exposing cells to CS-GAGs. As GAGs are highly soluble in aqueous media, it is likely that a significant portion of adsorbed species go into solution where soluble sulfated glycosaminoglycans can promote neurite outgrowth (Challacombe and Elam, 1997; Fernaud-Espinosa et al., 1994). We chose to covalently couple CS-GAGs to three-dimensional hydrogels to eliminate any confounding contribution from soluble CS-GAGs. Additionally, covalently coupled CS-GAGs in 2D render the substrates unfriendly to cell attachment which in turn prevents analysis of growth cone response. In this study, we avoid this consequence by trapping cells in the 3D gels containing covalently coupled CS-GAGs. Analysis with DMMB and FACE confirmed that all the CS-GAG in our gels was covalently coupled, and that no soluble CS-GAG was present (data not shown).

One caveat is that CS-GAGs that are purely CS-4, CS-6, or CS-4,6 are not available. However, commercially available CS-GAGs have a dominant CS-GAG sulfation pattern (Yoshida et al., 1989). Table 2 summarizes the correlation between available CS-GAGs (B, C, E, etc.) to their sulfation content. Even though the commercially available GAGs have more than one type of GAG, there are differential effects between neurite outgrowth in the particular gels. For example, the CS-E gel is much more inhibitory than the others gels except for the aggrecan species. The CS-E gel differs from CS-B and CS-C in that CS-E has a significant portion of CS-4,6S. Thus, it is reasonable to conclude that the increased inhibition is from the presence of this CS-4,6S epitope.

When growth cones encounter the same amount (0.5 mg/ml) of sulfated CS-GAGs per volume of agarose gel, different

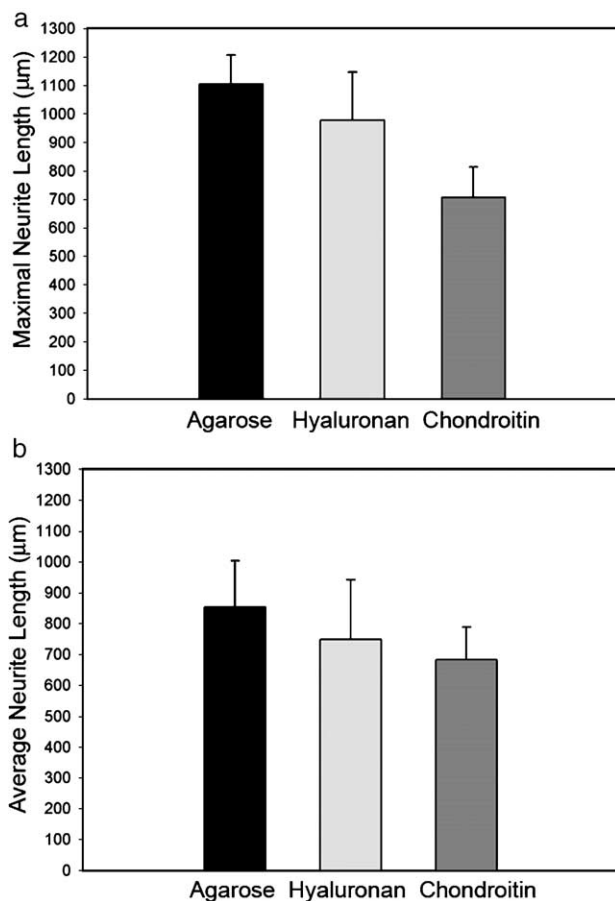


Fig. 8. Neurite outgrowth from E9 stage chick DRGs in various unsulfated GAG-agarose hydrogel systems. (a) The eight longest discernable neurites from each DRG were averaged and compared to other DRGs in each gel condition. Using a general linear ANOVA model, means for all of the gel conditions were statistically different ( $P < 0.05$ ). Using a Tukey 95% simultaneous confidence interval for pairwise comparisons, all pairwise comparisons were statistically significant ( $P < 0.05$ ). (b) The average neurite length for each DRG was determined using 50 lines from the explant to a line that connected the distinguishable growth cones of the neurites. Using a general linear ANOVA model, means for all of the gel conditions were statistically different ( $P < 0.05$ ). Using Tukey 96% simultaneous confidence interval for pairwise comparisons, all pairwise comparisons were statistically significant ( $P < 0.05$ ).

GAGs have significantly different degrees of inhibition. The inhibition in decreasing order of inhibition, CS-E (primarily CS-4,6) > aggrecan > CS-B (primarily DS-4) > CS-C (primarily CS-6). Although others have shown that proteoglycans consisting of GAGs with predominantly 6-sulfated *N*-acetyl-galactosamine are more inhibitory than a proteoglycan where the 4-sulfated *N*-acetyl-galactosamine predominates (Snow et al., 1990b), our study suggest that a more rigorous analysis of glycosaminoglycan content on PGs is necessary. FACE analysis of bovine aggrecan revealed that its glycosaminoglycan content is highly 4-sulfated (Table 2), and from our in vitro model, this aggrecan was equivalently inhibitory to CS-E (which is mostly 4,6-sulfated with a strong 4-sulfated component (Table 2)). Additionally, it is important to note that these data examine inhibition when equivalent amounts of sulfated CS-GAGs are presented to growth cones to determine their inhibitory potency.

In actual scar tissue, there is far greater CS-4,6 than in the normal, uninjured cortex. Therefore, the CS-4,6 GAG's effects are likely to be dominant due to their over expression in scar tissue in relation to their absence in normal, uninjured rat brain. Furthermore, other studies have shown that a specific astrocytic cell line, Neu 7, inhibits neuronal extension and produces more  $\Delta$ Di4,6S than a neurite permissive astrocytic cell line (A7) does (Properzi et al., 2005), suggesting that inhibition may be tied to this specific dual-sulfated CS-GAG moiety.

Our aggrecan gels, although containing lower sulfated GAG amounts than the CS-GAG gels (0.1 mg/ml of sulfated GAG compared to 0.5 mg/ml of sulfated GAG respectively), were very inhibitory. The only CS-GAG gel that was equally as inhibitory to the aggrecan gel was the CS-E gel. These data suggest that while various CS-GAGs are inhibitory, intact aggrecan remains more inhibitory per sulfated GAG than the other sulfated GAG gels. That intact aggrecan is more potently inhibitory suggests that there either might be other inhibitory domains in the CSPG such as the protein core (Lemons et al., 2003). Additionally, CSPGs may be far more efficient in presenting CS-GAGs to growth cones. It would be interesting to determine whether the CS-GAG profile seen in injured cortex can be correlated to specific CSPGs (e.g., Neurocan). Possibly, individual proteoglycans are responsible for the upregulation of the different CS-GAG seen in injured brain in vivo, and we are currently trying to unravel this issue in separate, ongoing studies.

Additionally, we used the agarose model to determine if the inhibitory character of CS-GAGs might be due to the structure of the GAG and not the presence of sulfation. When chondroitin (a CS-GAG without sulfation) is coupled to agarose, there is significant inhibition of neurite outgrowth (Fig. 8). If the native backbone structure of the CS-GAG is changed, as is the case when hyaluronan is used, neurite length is more similar to those neurites growing in agarose, implying that the sugar structure may be an important factor in inhibiting growth cones. Since chondroitin has the same backbone structure as the other sulfated CS-GAGs (CS-C and CS-E), the inhibitory effects may be structurally based, with specific degrees of sulfation enhancing inhibition.

Finally, we asked the question as to how representative the use of aggrecan as a substitute for scar-related CSPGs was. Based on our model, CS-4,6 dual-sulfated CS-GAG has an inhibitory character equal to that of aggrecan. However, aggrecan had no CS-4,6, the dominant GAG in scar tissue. This said, aggrecan in our system was very inhibitory and does have CS-4 and CS-6 sulfated GAGs which are also inhibitory. Unfortunately, it is technically impossible to study the inhibitory contribution of protein cores alone as all known digestion methods to remove GAGs leave GAG stubs behind.

We suggest a better understanding of the relative distribution and contribution of CS-GAGs in astro-glial scar tissue to inhibition of neurite extension is critical for the development of more specific strategies to overcome regenerative failure in the injured CNS.

## Experimental methods

### Generation of astroglial scar for analysis of CS-GAG profile

Isolation of glial scar tissue with little contamination with normal brain tissue is technically difficult in traditional models of CNS injury such as stab wounds. To circumvent this problem, we

used a model developed by Rudge and Silver (1990) and implanted nitrocellulose filters to generate gliotic scar tissue in a manner that afforded retrieval of scar tissue with little contamination with normal tissue. This model has the additional advantage of generating gliotic tissue that is representative of the normal *in vivo* response to CNS injury and allows for the collection of the variety of cells (i.e., astrocytes, fibroblasts, microglia/macrophages) and extracellular matrix molecules that comprise the glial scar (McKeon et al., 1991; Rudge and Silver, 1990). Nitrocellulose filters (Millipore, Bedford, MA) were placed bilaterally into the cerebral cortices of 45 day old female rats ( $n = 30$ ) using previously published methods (McKeon et al., 1999). In brief, the rats were anesthetized using a 1:1 mix of xylazine (20 mg/ml) and ketamine (100 mg/ml) (0.12 cc/100 g ip). The animals' heads were shaved and an incision was made down the middle of the scalp, exposing the skull. After the periosteum was cleaned, a dental drill was used to remove a rectangular piece of skull over each hemisphere. Next, a #11 scalpel blade was used to make an incision through the dura mater and in the cerebral cortex, into which a 3×3 mm piece of nitrocellulose was inserted. The incision was covered with Gelfoam and once all bleeding had stopped, the skin was stapled closed.

At 1 month post surgery, animals were sacrificed using sodium pentobarbital (100 mg/kg) and decapitated. The skull was removed and the filter was located by gently removing the overlying cerebral cortex. Well placed filter implants ( $n = 26$ ) were gently lifted from the grey matter of the cortex, rinsed in sterile saline, pooled into independent groups, and either placed into 1.5 ml microcentrifuge tubes, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  or placed into 200  $\mu\text{l}$  of lysis solution that contained 500 mM Tris (hydroxymethyl) aminomethane (Fisher Scientific, Bohemia, NY), 200 mM sodium chloride (Fisher Scientific, Bohemia, NY), and 1% triton X-100 (Sigma, St. Louis, MO) set to a pH of 8.0. The solution was pipetted for several minutes, aliquoted, and stored at  $-80^{\circ}\text{C}$  until subjected to the FACE analysis. Cortical tissue from the grey matter of uninjured age-matched rats was also removed and frozen to establish baseline expression of CS-GAGs. Non-implanted filters were also analyzed to determine if they had background levels of CS-GAGs. All surgical procedures and animal care was in accordance with guidelines established by the IACUC at Emory University.

#### *Glycosaminoglycan analysis using fluorophore-assisted carbohydrate electrophoresis (FACE)*

FACE analyses of filter-associated scar tissue, blank filters, and uninjured normal cortical tissue was performed using methods previously published (Calabro et al., 2000a,b,2001). Briefly, one set of implanted filters ( $n = 4$ ), control brain tissue, and blank filters were digested separately with proteinase K (250  $\mu\text{g}/\text{ml}$ ) dissolved in ammonium acetate buffer (Invitrogen Corporation, Calsbad, CA), and the samples were allowed to digest overnight. The four filters had a combined 9.6 mg of tissue (wet weight). Following proteinase K digestion, uninjured brain tissue was placed in aliquots so that each aliquot contained 4.8 mg of tissue (wet weight). Once the filter and uninjured brain tissue were digested, each sample was lyophilized and then taken to a volume of 300  $\mu\text{l}$  with ammonium acetate. To this, 1 ml of  $-20^{\circ}\text{C}$  absolute ethanol (Aaper, Shelbyville, KY) was added, agitated, and the sample stored overnight at  $-20^{\circ}\text{C}$ . The tubes were then placed into a centrifuge at  $10,000 \times g$  for 15 min. The supernatant was removed and saved. An additional 1 ml of absolute alcohol was

then added to the pellet, agitated, and then spun for 15 min at  $10,000 \times g$ . Following centrifugation, the 1.0-ml ethanol wash was then added to the supernatant fraction. The pellet fraction was then lyophilized dry and then taken to a volume of 300  $\mu\text{l}$  with ammonium acetate. One milliliter of absolute ethanol was added, and the sample was agitated and stored overnight at  $-20^{\circ}\text{C}$ . After double precipitation, the pellet was lyophilized to completion and re-suspended in ammonium acetate. The pellet samples from the filter samples were dissolved in ammonium acetate buffer, and then split into two equal fractions (each containing 4.8 mg of tissue), one to be digested with enzyme and one to be used as a no enzyme control. Individual aliquots of uninjured brain tissue were also enzyme digested or used as a no enzyme control. The enzyme controls were run to insure that the sample did not contain contaminants that might appear in the specific GAG lanes within the FACE gels. To the enzyme digested samples, hyaluronidase SD (Seikagaku, East Falmouth, MA) (10 mU/ $\mu\text{l}$ ) was added and allowed to digest for 1 h. After this hour, chondroitinase ABC (10 mU/ $\mu\text{l}$ ) (Seikagaku, East Falmouth, MA) was added and the digestion continued for an additional 3 h. After another hour, 0.5 U/ $\mu\text{l}$  glycoamylase (Seikagaku, East Falmouth, MA) was added to each sample as was 1.0 U/ $\mu\text{l}$  alkaline phosphatase (Sigma, St. Louis, MO). The samples were allowed to digest for a total of 4 h at  $37^{\circ}\text{C}$ . Samples were digested so that disaccharide sugars could be quantified and compared to other GAG samples. After enzymatic digestion, the ammonium acetate buffer was removed using a speed vacuum evaporator (model Speedvac SC110) (Thermo Electron Corp., Waltham, MA). Fresh 2-aminoacridone (AMAC) (Molecular Probes, Eugene, OR) was dissolved in a solution that contained by volume 85% DMSO (Aldrich, Milwaukee, WI) and 15% acetic acid (Aldrich, Milwaukee, WI). The AMAC solution was added to the entire sample, agitated, centrifuged, and then incubated for 10–15 min at room temperature. Fresh NaCNBH<sub>4</sub> (Aldrich, Milwaukee, WI) was prepared by placing this substance into ultrapure water. After incubation with AMAC, the NaCNBH<sub>4</sub> solution was added and allowed to incubate overnight at  $37^{\circ}\text{C}$ . Glycerol (Aldrich, Milwaukee, WI) was added to each sample, the samples agitated and spun down to ensure the sample was in the bottom of the tube. Glycerol-containing samples, metabolic sugars and GAG standards, and glucose standards were placed into a gel electrophoresis system that contained fabricated gels. Glucose was used to later quantify the amount of GAG in various tissue samples relative to known amounts of this sugar. Once samples were loaded, electrophoresis ensued on 1/40th of the sample (approximately 0.12 mg of tissue) until the standards were approaching the bottom of the gel. After gels were run, GAGs could be visualized and quantified as a result of the fluorescence capability of AMAC. Gel images were captured using a (12 bit Quantix Cooled CCD) camera. Images were analyzed using Gel-Pro Analyzer™ software (Media Cybernetics, Silver Spring, MD) and normalized based on weights of the tissue and filters. Fig. 9 schematically demonstrates the steps used to quantify GAG in the non-injured brain and in the filter implants. Samples were run on two separate gels to confirm initial results.

To confirm the initial results from the first set of filter samples and to quantify the CS-GAG distribution using enzyme chondro-6-sulfatase, filters were implanted into a second set of animals. After 30 days, the animals were euthanized and the second set of implanted filters was placed in lysis buffer. The lysate was subjected to the FACE procedure as previously described except chondro-6-sulfatase was used. For both the normal cortex and the

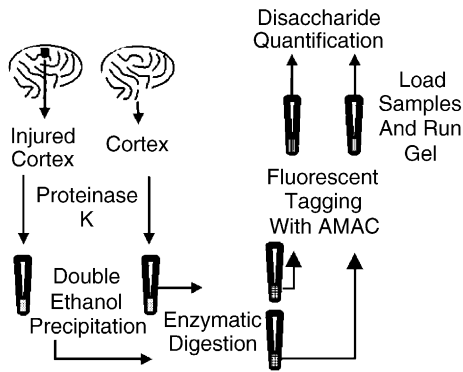


Fig. 9. The above represents a schematic describing the FACE procedure within our experimental design.

injured cortex samples, the samples were enzymatically treated as follows: (1) filter samples in which no enzyme was added; (2) filter samples in which the previous enzyme cocktail was used (hyaluronidase SD, chondroitinase ABC, glycoamylase, and alkaline phosphatase); and (3) filter samples in which the enzyme cocktail and chondro-6-sulfatase (Seikagaku, East Falmouth, MA) was used. The amount of lysate used in each condition approximated the amount of scar tissue from five filters. With the third condition, chondro-6-sulfatase digestion (10 mU/ $\mu$ l) was added after the typical 4-h digestion with the four enzymes mentioned previously and allowed to go overnight at 37°C. Following enzyme digestion, the ammonium acetate solution was removed using a speed vacuum and the samples were reacted with AMAC as specified above. The entire sample was reacted with AMAC, and 1/40th of the sample was run in an electrophoresis system. After the standards were allowed to run to the bottom of the gel, the intensity of the bands in the three samples was quantified and calibrated to picomolar quantities using glucose and maltose standards that each contained 25 pmol of substance. Amounts were then normalized based on the beginning mass of lysate used.

In order to confirm the initial FACE results, three additional control gels were run. The first gel was run to verify that chondro-6-sulfatase could shift standards to the appropriate position in a quantitative fashion. For this gel, 250 pmol of chondroitin sulfate standards ( $\Delta$ Di6S,  $\Delta$ Di4,6S,  $\Delta$ Di2,4,6S) (Seikagaku, East Falmouth, MA) were treated individually with and without chondro-6-sulfatase in the same manner as stated previously. The control (non-sulfatase-treated samples) and chondro-6-sulfatase samples were treated with AMAC and subjected to electrophoresis. Following electrophoresis, the AMAC bands were quantified to determine the conversion efficiency of the chondro-6-sulfatase on the various standards. A second gel was run to verify that chondro-6-sulfatase would not shift a blank sample that had AMAC contaminant running in the  $\Delta$ Di6S position. Two empty tubes were taken and 100  $\mu$ l of ammonium acetate buffer was added to each tube. The solution was subjected to 4 h of incubation at 37°C but was not treated with the normal enzyme cocktail. Following the 4 h of incubation, one tube was subjected to an overnight digestion with chondro-6-sulfatase. Following digestion, both tubes were subjected to AMAC staining and electrophoresis. A third gel was run to verify that the band seen in the enzyme digested filter samples was  $\Delta$ Di4,6S by mixing in known  $\Delta$ Di4,6S standard with enzyme digested filter samples. 250 pmol of  $\Delta$ Di4,6S was

stained with AMAC and added to an injured cortex sample that had been enzymatically digested (but not with chondro-6-sulfatase) and stained with AMAC. This mixed sample was then subjected to electrophoresis as was the individual  $\Delta$ Di4,6S standard and injured cortex samples.

Since some sulfated glycosaminoglycan data were available from previous published reports (Yoshida et al., 1989), the sulfated GAG profile for CS-B and aggrecan was determined by using the FACE analysis methods described above. These two samples underwent the same FACE analysis as mentioned previously.

#### Fabrication of CS-GAG or CSPG-immobilized agarose gels

CS-GAG chains were attached to agarose using photocrosslinking chemistry (Chen et al., 1997) (Fig. 10). Eighteen milligrams of either 6-sulfated CS-GAG (CS-C), 4,6-sulfated CS-GAG (CS-E), hyaluronan, chondroitin, (Seikagaku, East Falmouth, MA), 4-sulfated dermatan sulfated glycosaminoglycan (DS-GAG) (CS-B) (Sigma, St. Louis, MO), or aggrecan (supplied by Dr. David Carrino, Department of Biology, Case Western Reserve University) was dissolved into 20 ml of distilled, deionized water with 4 mg of 4-azidoaniline (Aldrich, St. Louis, MO) and 10 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC) (Sigma, St. Louis, MO). After 3 h of stirring at 4°C, the solution was removed and placed into cellulose ester dialysis tubing with a molecular weight cut off of 10,000 (Spectrum Laboratories Inc., Rancho Dominguez, CA) to remove unbound 4-azidoaniline and EDAC. Once unbound constituents were removed, the product was frozen in a -80°C freezer and lyophilized (Labconco Corporation, Kansas City, MO). The GAG or PG with crosslinker was shredded to increase surface area for subsequent dissolving procedures and then added to 3 ml of 1.5% (w/v) SeaPrep agarose (Cambrex, Rockland ME) in phosphate-buffered saline (PBS) (Invitrogen Corporation, Carlsbad, CA), mixed thoroughly, and mildly heated if the lyophilized powder did not dissolve at room temperature. Once the constituent dissolved, another 3 ml of PBS was added until the final concentration of agarose in PBS was 0.75% (w/v). To bind the conjugate to the agarose, the solution was placed into a

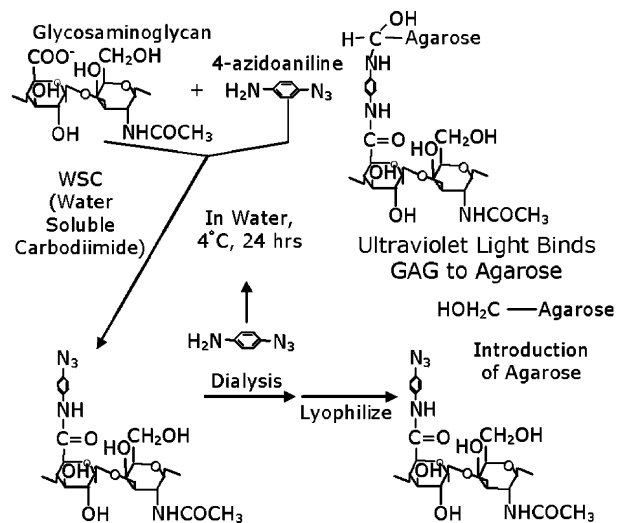


Fig. 10. Schematic of the coupling chemistry used to couple GAGs to agarose gels.

Pyrex glass Petri dish (6 cm diameter) (Corning Incorporated, Big Flats, NY) and placed under a long wave ultraviolet lamp (Blak-ray, model B-100AP) (UVP, Upland, CA) for 2 min. After irradiation, the liquid was cooled to 4°C, transforming the agarose into its gel state. Unbound CS-GAG (CS-B, CS-C, or CS-E), hyaluronan, chondroitin, or aggrecan was removed by adding 5 ml of chilled (4°C) PBS to the gel with agitation (100 rpm) via an orbital shaker (Lab-line Instruments, Inc., Melrose Park, IL) for approximately 5 min. The PBS was removed and the above washing steps were repeated four more times, with each wash lasting 5 min. Gels were then heated until becoming liquid and sterile filtered using a 0.2- $\mu$ m filter (Nalge Company, Rochester, NY).

#### *Quantification of sulfated CS-GAG or PG immobilized to agarose*

Once covalently immobilized CS-GAG gels were washed, the amount of sulfated CS-GAG/volume of gel was quantified using 1,9-dimethyl-methylene blue (DMMB) (Aldrich, Milwaukee, WI) (Dillon et al., 2000) to measure the sulfur content within our glycosaminoglycan samples colorimetrically. The amount of CS-GAG/volume of gel was quantified for the immobilized CS-B, CS-C, CS-E, and aggrecan systems. To generate a standard curve, CS-C was dissolved in SeaPrep agarose (1% w/v in PBS) at a concentration of 1 mg/ml and used to create standards of 0.6, 0.2, and 0.1 mg/ml of CS-C, in triplicate, and brought to a final volume of 50  $\mu$ l with PBS. A 50- $\mu$ l sample of agarose without CS-GAG was used as the 0.0 mg/ml condition. To these standards, 2.5 ml of DMMB dissolved in deionized, distilled water was added and mixed using a vortex (Fisher Scientific, Bohemia, NY). Using a spectrophotometer (Shimadzu, Model UV-1601, Kyoto, Japan), absorbance at 525 nm was quantified. Averages for the standards were computed and a calibration curve for the CS-C condition was constructed, relating CS-GAG concentration to absorbance at 525 nm. Next, 50  $\mu$ l of liquefied gel with immobilized CS-GAG/PG was added to 2.5 ml of DMMB, mixed, placed into the spectrophotometer to determine the sulfated CS-GAG concentration within the CS-B, CS-C, CS-E, and aggrecan systems. Since the calibration involved only the CS-C species, the CS-B and CS-E gels were normalized based on their known sulfation content as supplied by the chemical supplier or as determined through FACE analysis of the compound. After the CS-B and CS-E gels were normalized, the sulfated CS-GAG concentration of the CS-B, CS-C, and CS-E gels were diluted with sterile 1% agarose gels in PBS until their sulfated CS-GAG concentration was 0.5 mg/ml.

#### *Quantification of chondroitin and hyaluronan immobilized to agarose*

After chondroitin and hyaluronan were bound to agarose and sterile filtered, 100  $\mu$ l of each of the liquefied gel species was placed into corresponding pre-weighed microcentrifuge tubes. After adding the gels to the microcentrifuge tubes, the weight of the tube plus the gel was measured. The gels were then melted to 70°C using a heating block (Isotemp 125D) (Fisher Scientific, Pittsburgh, PA). Once the gels were melted, 2  $\mu$ l of 50 $\times$   $\beta$ -agarase buffer (Cambrex, Rockland, ME) was added and mixed. The gel was then allowed to cool to 45°C. One unit of  $\beta$ -agarase enzyme (Cambrex, Rockland, ME) was added to the gel solution and

allowed to digest overnight. The resulting digested gels were subjected to FACE analysis to quantify the amount of hyaluronan or chondroitin per wet weight of gel.

#### *Quantification of sulfated GAG concentration in uninjured brain*

A small piece of rat cortex (66.8 mg wet weight) was weighed and then subjected to proteinase K digestion as mentioned previously. Once proteinase K digestion was complete, the GAG species within the brain was precipitated out using absolute ethanol that was maintained at a temperature of -20°C. After two ethanol precipitation steps, any remaining solvent was lyophilized away. The dry pellet was then re-suspended in 200  $\mu$ l of ammonium acetate buffer. From this solution, 10  $\mu$ l of precipitate was placed into a 96-well plate, to which 40  $\mu$ l of ammonium acetate solution was added. To create concentration standards, 5 mg of CS-B was dissolved into 25 ml of ammonium acetate solution, creating a 200- $\mu$ g/ml stock solution. Subsequent standards were made (in 25  $\mu$ g/ml increments) by diluting the stock solution. A volume of 50  $\mu$ l from each standard concentration was added to a 96-well plate. Each standard and the sample were run in triplicate. To each sample and standard, 150  $\mu$ l of DMMB solution was added. Absorbance readings were gathered using a plate reader (BioTek Instruments, Inc., Winooski, VT) whose absorbance was set at 525 nm. For each standard and sample, absorbance readings were averaged. A standard curve was created based on the absorbance readings, and the concentration of sulfated GAG within the brain based on CS-B was determined. To determine if the sulfated GAG concentration within brain was dependent on the type of commercial-sulfated GAG used to create the calibration curve, CS-C standards were also prepared and quantified in the same manner as that for the CS-B standards.

#### *Culture of E9 DRGs in CS-GAG and control gels*

Culture systems were created by taking 48-well plates (Corning Incorporated, Corning, NY) and precoating the surface with 125  $\mu$ l of a specific liquefied gel. The plates were then wrapped with parafilm (American National Can, Menasha, WI) and placed into a 4°C environment to solidify the gel. Dorsal root ganglion (DRG) explants were removed from the lumbosacral regions of E9 stage chickens and placed into chilled calcium/magnesium-free Hanks Balanced Salt Solution (HBSS-CMF) solution (137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO<sub>3</sub>, 15 mM C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.43 mM KH<sub>2</sub>PO<sub>4</sub>). After dissection, explants were cut into halves and placed via forceps into 0.5 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) that contained 125  $\mu$ l of either CS-B, CS-C, CS-E, CSPG, chondroitin, or hyaluronan gel. Each gel, in its liquid state, with explants was then pipetted onto the 48-well plate, in the wells that were precoated with gel. The plates were wrapped with parafilm and placed into a 4°C environment for approximately 15 min to allow for gelation. Once the gel solidified, 500  $\mu$ l of Neurobasal™ medium (Invitrogen™ Life Technologies, Carlsbad, CA) supplemented with non-serum additive B-27 (Invitrogen™ Life Technologies, Carlsbad, CA) and 50 ng/ml of nerve growth factor (mNGF, 2.5S) (Alomone Labs, Jerusalem) were added on top of the gels in each well and the plates were transferred to an incubator (37°C, 95%

humidity, 5% CO<sub>2</sub>) and cultured for 48 h. All procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at Case Western Reserve University and the Georgia Institute of Technology.

#### Measurement of DRG neurite length

After 48 h of culture time, cultures were placed under a light microscope and the longest neurites within each gel condition were identified using our image analysis system described below. Cultures were analyzed using either a TE300 Eclipse inverted microscope (Nikon, Melville, NY) or an Axiovert 200 M microscope (Carl Zeiss, Inc., Thornwood, NY) and images were captured using either a Magnafire (Optronics, Goleta, CA) or a Retiga EXi Fast 1394 digital camera (Q-imaging, Burnaby, B.C. Canada). Neurite lengths were computed from the captured images by Image Pro Express or Image Pro Plus (Media Cybernetics, Carlsbad, CA) software and calibrated using a stage micrometer (Fisher Scientific, Pittsburgh, PA). Neurite length was traced from the edge of the explant, along the neurite, and then to the end of the neurite if the neurite was discernable from growth cone to explant. Neurite lengths were computed only from those DRGs that had neurites emanating in all directions. Additionally, lengths computed were two-dimensional projections and thus are not the actual length in the three-dimensional environment. Therefore, our length measurements underestimate the actual length of the neurites. For each DRG, 8–15 of the longest neurites were identified and their length measured. After these lengths were measured, the eight longest neurites from the DRG were isolated and used to compare neurite lengths between agarose and the various CS-GAG, PG, chondroitin, or hyaluronan gels.

In addition to the longest neurite length measurements, the average neurite length on each DRG was computed. To determine the average neurite length for a particular DRG, a 90° arc was traced connecting the growth cones farthest away from the DRG. Then, 50 straight lines were drawn connecting the above line to the DRG explant as it was technically difficult to trace individual neurites due to their high density in our 3D cultures. When comparing the longest DRG neurite lengths or average neurite lengths between agarose and the sulfated CS-GAG or PG, at least four DRGs were used. When comparing DRG neurite lengths or average neurite lengths between agarose and unsulfated GAGs, at least three DRGs were used. For statistical analysis, a general linear ANOVA model was used to compare mean values of the different conditions. Pairwise comparisons were conducted using Tukey 95% simultaneous confidence intervals (Minitab Inc., State College, PA).

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