MODULATION OF CSPG SULFATION PATTERNS THROUGH SIRNA SILENCING OF SULFOTRANSFERASE EXPRESSION TO PROMOTE CNS REGENERATION

A Thesis
Presented to
The Academic Faculty

by

Mary Angela Millner

In Partial Fulfillment
of the Requirements for the Degree
Masters of Science in Bioengineering in the
Department of Biomedical Engineering/ College of Engineering

Georgia Institute of Technology
August 2008

MODULATION OF CSPG SULFATION PATTERNS THROUGH SIRNA SILENCING OF SULFOTRANSFERASE EXPRESSION TO PROMOTE CNS REGENERATION

Approved by:

Dr. Ravi Bellamkonda, Advisor
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Michelle LaPlaca
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Robert McKeon
Department of Cell Biology
Emory University

Date Approved: July 2nd, 2008

ACKNOWLEDGEMENTS

To begin I wish to thank my research advisor, Dr. Ravi Bellamkonda, for giving me the wonderful opportunity to learn from him. I would also like thank to the many members of the Bellamkonda Lab that I've been fortunate to work with over the last several years. I've learned so much from each and every one of you. I would especially like to acknowledge Abhiruchi Agarwal, Anjana Jain, and Vivek Mukhatyar for teaching me so much about their culture and making me an honorary Indian. Thanks to all of the amazing friends that I've met during my time spent at Georgia Tech, especially Adam, Allison, Andrea, C-Dub, Hillary, Gustavo, Maxine, MCL, Neil, Nimisha, Sarah S, and Stacey. I survived graduated school because of the experiences that we shared. And lastly, I would like to thank John, my best friend and my personal scientific advisor. I couldn't have done this without you.

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LIST OF ABBREVIATIONS

SCI	Spinal Cord Injury
CNS	Central Nervous System
PNS	Peripheral Nervous System
MAG	Myelin Associated Glycoprotein
OMgp	Oligodendrocyte Myelin Glycoprotein
GFAP	Glial Fibrillary Acidic Protein
BBB	Blood Brain Barrier
CSPG	Chondroitin Sulfate Proteoglycan
CS-GAG	Chondroitin Sulfate Glycosaminoglycan
PAPS	3' Phosphoadenosine-5' Phosphosulfate
GlcUA	Glucuronic Acid
GalNAc	Galactosamine
Xyl	Xylose
Gal	Galactose
XT	Xylose Transferase
GalT	Galactose Transferase
GlcUAT	Glucuronic Acid Transferase
GalNAcT	GalNAc Transferase
ChSy	Chondroitin Synthase
ChPF	Chondroitin Polymerizing Factor
CSST	Chondroitin Sulfate Sulfotransferase

C4ST	Chondroitin 4-Sulfotransferase
C6ST	Chondroitin 6-Sulfotransferase
C46ST	N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase
UST	Uronyl-2-Sulfotransferase
ChABC	Chondroitinase ABC
siRNA	Small Interfering RNA
dsRNA	Double Stranded RNA
shRNA	Short Hairpin RNA
RNAi	RNA Interference
RISC	RNA-Induced Silencing Complex
CM	Conditioned Media

SUMMARY

Injury to the central nervous system (CNS) results in the formation of a highly inhibitory glial scar consisting mainly of chondroitin sulfate proteoglycans (CSPGs). CSPGs are comprised of a protein core with covalently attached chondroitin sulfate glycosaminoglycan (CS-GAG) side chains. CSPGs and CS-GAGs have been implicated in the regenerative failure of the CNS, though the mechanism underlying inhibition is unclear. Sulfation affects both the physical and chemical characteristics of CS-GAGs and, therefore, it has been hypothesized that certain sulfation patterns are more inhibitory than others. To investigate this hypothesis, specific chondroitin sulfate sulfotransferases (CSSTs), the enzymes responsible for CS-GAG sulfation, were knocked down in vitro using siRNA. C4ST-1, C4ST-2, and C46ST were chosen as targets for gene knockdown in this study based on their expression in neural tissue and the extent of inhibition caused by their respective CS-GAG. It was hypothesized that transfection of primary rat astrocytes with siRNAs designed to prevent the expression of C4ST-1, C4ST-2, and C46ST would decrease specific sulfation patterns of CSPGs, resulting in improved neurite extension in a neurite guidance assay. Through optimization of siRNA dose, astrocyte viability was maintained while successfully knocking down mRNA levels of C4ST-1, C4ST-2, and C46ST and significantly reducing total levels of secreted CS-GAGs. However, no increase in the incidence of neurite extension was observed using conditioned media collected from siRNA transfected astrocytes compared to non-transfected controls. These data suggest that sulfation does not contribute to CSPG-mediated neurite inhibition, though further investigation is necessary to confirm these

findings. Significantly, this work has established a paradigm for investigating the role of CSPG sulfation patterns in CNS regeneration.

CHAPTER 1

INTRODUCTION

Background

Spinal Cord Injury

In the United States alone there are an estimated 12,000 new cases of spinal cord injury (SCI) a year, with approximately 250,000 individuals living with an injury. The majority of SCIs result in permanent loss of muscle function and physical sensation below the area of injury, significantly reducing the quality of life and lowering the life expectancy of the individual. Currently, there is no clinical treatment to regenerate the severed or degenerated axons of an injured spinal cord, only ways to prevent further damage from occurring. Furthermore, with medical expenses and patient care costing between $200,000 and $700,000 in the first year and up to $3 million in a lifetime, SCI can be a financial hardship on both the individual and his or her family (www.spinalcord.uab.edu, 2008).

Regenerative Failure in the CNS

Following injury to the central nervous system (CNS) axons fail to regenerate. Historically, this was considered to be a property inherent to neurons of the CNS (Clark, 1943) however, when neurons derived from the CNS are implanted in the peripheral nervous system (PNS), where spontaneous regeneration does occur, extensive axon sprouting is observed (Richardson et al., 1980; David and Aguayo, 1981). Furthermore,

while limited neurite sprouting has been observed in the injured CNS (Liu and Chambers, 1958), the growth cones quickly become dystrophic and are incapable of entering the lesion site (Misgeld and Kerschensteiner, 2006; Misgeld et al., 2007). Hence, it is now believed that regenerative failure is due to the extrinsic molecular environment at the site of injury, rather than the intrinsic nature of the cells. In particular, there are several classes of molecules locally released at the site of injury that are potently inhibitory to neuronal regeneration.

Myelin Related Inhibitors

Neurite inhibition has been largely attributed to the release of several molecules from disrupted CNS myelin, commonly referred to as the myelin related inhibitors. These molecules include myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), and oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002). Surprisingly, it was found that the neuronal receptor to Nogo, NgR, also binds to MAG and OMgp, suggesting that all three function through a common pathway (Fournier et al., 2001; Domeniconi et al., 2002; Wang et al., 2002). Such findings have made myelin associated inhibitors promising therapeutic targets for promoting CNS regeneration. In vivo, neutralization of Nogo, using anti-Nogo antibodies (Bregman et al., 1995; Fouad et al., 2004) or NgR receptor antagonists (GrandPre et al., 2002), has resulted in significantly improved axon sprouting. Despite this, little or no regeneration occurs in Nogo (Simonen et al., 2003)[21], MAG (Bartsch et al., 1995), or NgR (Kim et al., 2004) knockout models (an OMgp KO does not exist), particularly in

the cortical spinal tract. For this reason, it is a generally accepted that although the myelin related inhibitors impede neurite outgrowth, they are not the key inhibitory components.

Scar Related Inhibitors

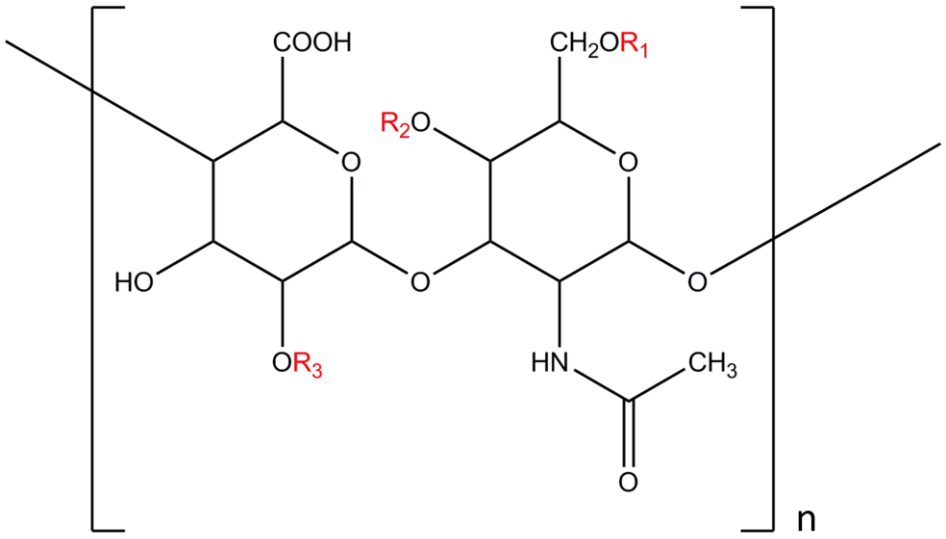
Despite the effects of myelin related inhibitors on neurite outgrowth, the primary cause of regenerative failure is believed to be the formation of the highly inhibitory glial scar, which presents both biochemical and physical barriers. Disruption of the blood brain barrier (BBB) and initial cell death following injury results in substantially increased levels of cytokines and growth factors in the area, activating astrocytes (reactive astrocytes) (Eng et al., 1987) and microglia (activated microglia) (Kreutzberg, 1996). Consequently, production of the intermediate filament protein, glial fibrillary acidic protein (GFAP), is immediately upregulated in reactive astrocytes, causing the cells to become hypertrophic and extend filopodia, thereby generating a barrier that participates in the repair of the BBB, sequestering the injured area and protecting healthy tissue from damage (Bush et al., 1999; Faulkner et al., 2004). Unfortunately, this barrier is not permissive to axon growth, and, in turn, prevents functional recovery after injury. Hence, the glial scar acts as both a physical and chemical barrier to axonal regeneration; the dense meshwork of hypertrophic cells and extracellular matrix physically impede growth cone migration (Windle and Chambers, 1950), while inhibitory molecules secreted by reactive astrocytes and activated microglia, namely chondroitin sulfate proteoglycans (CSPGs) (Fawcett and Asher, 1999), generate a biochemical barrier to axonal regeneration (McKeon et al., 1991).

Chondroitin Sulfate Proteoglycans

Structure and Synthesis

CSPGs consist of a protein core with a variable number of chondroitin sulfate glycosaminoglycan (CS-GAG) side chains covalently attached to serine residues. CS-GAGs are unbranched polysaccharide chains comprised of repeating disaccharides of glucuronic acid (GlcUA) and galactosamine (GalNAc). These chains can be further modified by the transfer of sulfate groups from 3' phosphoadenosine-5' phosphosulfate (PAPS) onto the disaccharide's hydroxyl groups (Silbert and Sugumaran, 2002). Monosulfated GAGs result from the sulfation at the 4 or the 6 carbon of GalNAc (CS-4 and CS-6, respectively) and disulfated GAGs result from the sulfation at either the 2 carbon of GlcUA and the 6 carbon of GalNAc (CS-2,6), or at both the 4 and the 6 carbons of GalNAc (CS-4,6) (Figure 1.1).

It is difficult to isolate pure forms of the specific CS-GAGs given that in nature individual CS-GAG chains usually consist of a combination of the various disaccharides. The commercially available CS-GAGs are mixtures of the dissacharides, though CS-A and CS-B (or dermatan sulfate) consist mostly 4-sulfated chains, CS-C consists mostly 6-sulfated chains, CS-D consists mostly of 2,6 sulfated chains, and CS-E consists mostly of 4,6 sulfated chains. In dermatan sulfate the GlcUA is epimerized to iduronic acid (Silbert and Sugumaran, 2002).



	R_1	R_2	R_3
Chondroitin-0-sulfate	H	H	H
Chondroitin-4-sulfate	H	SO_3H	H
Chondroitin-6-sulfate	SO_3H	H	H
Chondroitin-2,6-sulfate	H	SO_3H	SO_3H
Chondroitin-4,6-sulfate	SO_3H	SO_3H	H

Figure 1.1. Chondroitin Sulfate Disaccharide Unit. CS-GAG chains are comprised of repeating disaccharides of glucuronic acid and galactosamine. The disaccharide is sulfated at R_1, R_2, or R_3 depending on the type of CS-GAG as demonstrated in the table.

The biosynthesis of CS-GAG chains occurs with the aid of multiple enzymes (Figure 1.2), beginning with the addition of xylose (Xyl) to a serine residue on the core protein, followed by the addition of two galactose (Gal) residues, and a glucuronic acid residue (GlcUA), forming the tetrasaccharide linkage region (Ser[Xyl-Gal-Gal-GlcUA]-) common to *all* chondroitin, dermatan, and heparan sulfates. Each of these sugars is successively added by a specific glycosyltransferase (xylose transferase-I (XT-I) (Gotting et al., 2000), galactose transferase-I (GalT-I) (Almeida et al., 1999), galactose transferase-II (GalT-II) (Bai et al., 2001), glucuronic acid transferase-I (GlcUAT-I) (Kitagawa et al., 1998), respectively). Next, a fifth glycosyltransferase, GalNAc transferase-I (GalNAcT-I), adds a GalNAc residue, thereby committing the proteoglycan to becoming a CS-GAG (Silbert and Sugumaran, 2002). Chain polymerization continues by the addition of alternating GlcUA and GalNAc residues through a complex consisting of chondroitin synthase (ChSy) and chondroitin polymerizing factor (ChPF). ChSy is responsible for synthesizing the disaccharides (Kitagawa et al., 2001) whereas ChPF is responsible for chain polymerization (Kitagawa et al., 2003).

During chain polymerization, CS-GAGs are further modified by the sulfation of the disaccharides with the aid of specific chondroitin sulfate sulfotransferases (CSSTs) (Figure 1.3). 4 and 6 sulfation occurs through one of three chondroitin 4-sulfotransferases (C4ST-1 (Hiraoka et al., 2000; Mikami et al., 2003), C4ST-2 (Hiraoka et al., 2000; Mikami et al., 2003), or C4ST-3 (Evers et al., 2001) or one of two chondroitin 6-sulfotransferases (C6ST-1 (Fukuta et al., 1995; Uchimura et al., 1998) or C6ST-2 (Kitagawa et al., 2000)), respectively. N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (C46ST) sulfates the 6 hydroxyl of a preexisting CS-4 to form CS-4,6

(Ito and Habuchi, 2000), and uronyl-2-sulfotransferase (UST) sulfates the 2 hydroxyl of a preexisting CS-6 to form CS-2,6 (Kobayashi et al., 1999).

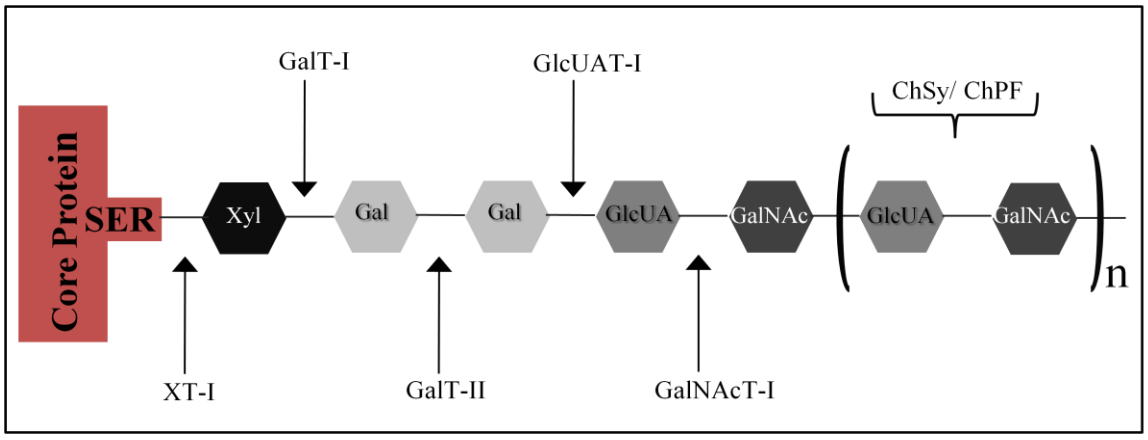


Figure 1.2. Enzymes in CS-GAG Chain Biosynthesis. Many enzymes aid in the biosynthesis of CS-GAG chains. XT-1 initiates synthesis by adding a Xyl to a Ser on the core protein. GalT-I, GalT-II, and GlcUAT-I then successively add 2 Gals and GlcUA, respectively, forming the tetrasaccharide linkage region (Ser-[Xyl-Gal-Gal-GlcUA]) common to all proteoglycans. Next, GalNAcT-I adds a GalNAc, thereby committing the proteoglycan to becoming a CS-GAG. Chain polymerization continues by the addition of alternating GlcUA and GalNAc residues through a complex consisting of ChSy, which is responsible for synthesizing the disaccharides, and ChPF which is responsible for chain polymerization.

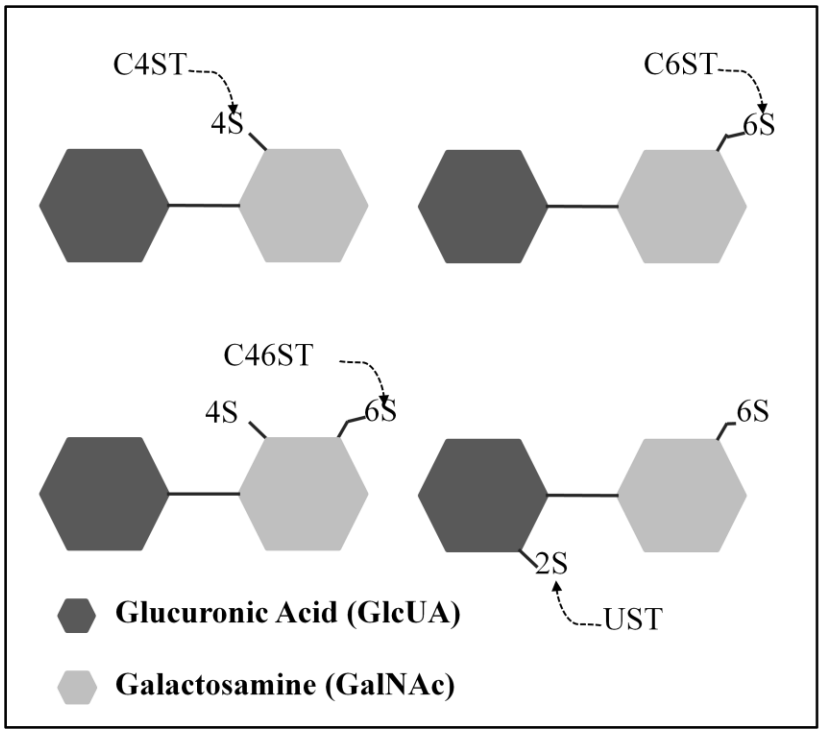


Figure 1.3. Chondroitin Sulfate Sulfotransferases. Sulfates are added to each position of chondroitin sulfate with the aid of specific CSSTs. 4-sulfation occurs through one of three chondroitin 4-sulfotransferases (C4ST-1, C4ST-2, or C4ST-3). 6- sulfation occurs through one of two chondroitin 6-sulfotransferases (C6ST-1 or C6ST-2). N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (C46ST) sulfates the 6 hydroxyl of a preexisting CS-4 to form CS-4,6. Uronyl-2-sulfotransferase (UST) sulfates the 2 hydroxyl of a preexisting CS-6 to form CS-2,6.

Biological Functions

The ability for cells to create such diverse structures through modifying chain length and CS-GAG disaccharide composition has resulted in several distinct biological functions of CSPGs. These molecules have been implicated in growth factor signaling (Deepa et al., 2002), wound healing (Yeo et al., 1991), inflammation (Taylor and Gallo, 2006), cell division (Bechard et al., 2001), and tissue morphogenesis (Hwang et al.,

2003). In cartilage CSPGs are vital for the tissue's ability to resist high compressive loading. This is due to the net negative charge of the CS-GAGs, which allows them to hold in water molecules causing the tissue to create an osmotic swelling pressure (Bayliss et al., 1999). In the developing CNS, CSPGs act as repulsive cues in cell migration (Nishizuka et al., 1996) and define barriers for extending neurites (Brittis et al., 1992). Additionally CSPGs appear to play an important role in CNS plasticity and are a major component of perineuronal nets (Celio and Blumcke, 1994). Following injury to the CNS there is a significant upregulation of CSPG production, predominantly by reactive astrocytes, forming the highly inhibitory glial scar.

CSPGs often function through their interactions with various growth factors, cell adhesion molecules, enzymes, and ECM components. The ability for CSPGs to interact with these molecules is dependent on their specific CS-GAG content.

CSPGs in Regenerative Failure

The inhibitory role of CSPGs in axonal regeneration has been clearly demonstrated both in vitro (McKeon et al., 1995; Meiners et al., 1995) and in vivo (Zhang et al., 2001), however, the mechanism by which inhibition occurs is poorly understood. In particular, there is much debate over whether the CS-GAGs or the protein core contribute more to inhibition. Certain CSPGs, such as brevican (Yamada et al., 1997), phosphacan (Sango et al., 2003) and neurocan (Talts et al., 2000; Sango et al., 2003), lose at least some of their inhibitory capabilities following treatment with chondroitinase ABC (chABC), suggesting a CS-GAG mediated inhibition, whereas other CSPGs, such as NG2 (Dou and Levine, 1994) and versican (Schmalfeldt et al., 2000), do

not, suggesting a protein core mediated inhibition. Furthermore, studies investigating the effects of isolated CS-GAGs on neurite outgrowth in vitro have produced conflicting results. Some have reported CS-GAGs to be inhibitory to neurite outgrowth (Brittis and Silver, 1994; Becker and Becker, 2002), whereas others have observed the opposite (Faissner et al., 1994; Fernaud-Espinosa et al., 1994; Challacombe and Elam, 1997; Nadanaka et al., 1998; Clement et al., 1999). An explanation for this inconclusive data is that in these experiments no distinction was made between soluble and substrate bound CS-GAGs, and previously it has been shown that substrate bound CSPGs inhibit neurite outgrowth whereas soluble CSPGs do not (Snow et al., 1996). Because of their highly hydrophilic nature, CS-GAGs are not readily immobilized via adsorption, and are therefore difficult to study in a substrate bound form. When neurite extension was measured from cells grown in 3D hydrogels with CS-GAGs covalently attached (ensuring CS-GAG immobilization) all three CS-GAGs explored (CS-B, CS-C, CS-E) demonstrated an inhibitory effect (Gilbert et al., 2005). Furthermore, the extent of inhibition was dependent on sulfation position and degree of sulfation. The greatest contributor to inhibition was CS-E, which was comparable to the full CSPG, followed by CS-B (Gilbert et al., 2005). These data suggests that not only do the CS-GAGs contribute to CSPG-mediated inhibition, but that sulfation also plays a major role in this function.

CS-GAG Composition in Development and After Injury

The composition of CS-GAG disaccharides changes throughout development. Specifically, the percentage of CS-GAGs either non-sulfated or 4-sulfated increases from

84% in embryonic rat cortical tissue to 96% in adult tissue and the percentage of 6-sulfated CS-GAGs decreases from 14% in embryonic tissue to 2% in adult tissue (Properzi et al., 2005). Additionally, CS-GAG disaccharide composition changes after injury. Most notably, CS-4,6, which is nearly undetectable in uninjured tissue is significantly upregulated in adult cortical scar tissue (Gilbert et al., 2005). Given that the ability for CNS neurons to regenerate decreases throughout development (Dusart et al., 1997) and that the environment in the CNS after injury is highly inhibitory, this suggests that the inhibitory properties of CSPGs may be dependent on disaccharide composition. Specifically, CS-4 and CS-4,6 may be particularly inhibitory given that the percentage at which they are present increases as regenerative capacity decreases. Collectively, these data corroborate previous findings indicating that CS-4,6 is the most inhibitory CS-GAG followed by CS-4 (Gilbert et al., 2005).

Chondroitinase ABC

Chondroitinase ABC (chABC) is an enzyme purified from the bacterium *Proteus vulgaris* that degrades chondroitin sulfate (Yamagata et al., 1968). ChABC consists of an endoeliminase that depolymerizes chondroitin sulfate and an exoeliminase that breaks down tetra- and hexasaccharides into disaccharides (Hamai et al., 1997). The ABC in the name refers to CS-A, CS-B, and CS-C, though the enzyme is also effective in cleaving CS-D, and CS-E.

Therapeutic Applications of chABC

The delivery of chABC in vivo has resulted in a promising increase in axonal sprouting (Moon et al., 2001; Bradbury et al., 2002). However, several disadvantages are associated with the use of this enzyme clinically. First, the cleavage of CS-GAGs by chABC is non-specific and, consequently, all CSPGs at the site are degraded, including those necessary for proper CNS function, namely CSPGs involved in the formation of perineuronal nets (Bruckner et al., 1998). In addition, chABC is incapable of digesting the initial tetrasaccharide that is added to the core protein to begin CS-GAG chain synthesis, resulting in the presence of an "inhibitory stub" (Caterson et al., 1985; Lemons et al., 2003). Moreover, the enzymatic activity of chABC is short lived in vivo, and therefore multiple injections are necessary to achieve sufficient degradation (Tester et al., 2007). Finally, because chABC is a bacterial enzyme (Yamagata et al., 1968) and no mammalian equivalents currently exist, repeated dosing necessary for effective cleavage may trigger an immune response.

siRNA

Mechanism

Small interfering RNAs (siRNAs) are short nucleic acid sequences, 21 to 23 base pairs in length, which are capable of post transcriptional gene silencing by inducing the degradation of complimentary mRNA sequences. siRNA is an integral component of the RNA interference (RNAi) pathway, an evolutionarily conserved mechanism that protects the genome from foreign genetic elements. More specifically, this pathway degrades potentially harmful double stranded RNA (dsRNA), such as those found in cells infected

by a virus. When dsRNA is detected in a cell, Dicer, a cytoplasmic ribonuclease III-like enzyme, cleaves it into 21 to 23 nucleotide fragments (siRNAs). These siRNA then bind to a multiprotein complex called RNA-induced silencing complex (RISC), where the dsRNA is linearized and the strands are separated. The single stranded RNA bound to RISC, or the guide strand, then complexes with complimentary mRNA. The mRNA is cleaved by Argonaute 2, an enzyme present in RISC, thereby preventing translation of the transcript. Once mRNA degradation is complete, the guide strand remains bound to RISC, allowing for the complex to bind with additional mRNA, thereby amplifying the degradation of the target mRNA (Figure 1.4) (Aagaard and Rossi, 2007).

Applications of siRNA

Recently, the RNAi pathway has proven to be a powerful tool in studying the function of specific genes both in vitro (Scherr et al., 2003) and in vivo (Lingor and Bahr, 2007). Cells can be infected with a vector that is processed into short hairpin RNA (shRNA) containing 21-base pair dsRNA which are cleaved by Dicer into siRNA. Alternatively, synthetic siRNA can be transfected into cells which thereby directly activates RISC. The success in silencing specific genes has sparked much interest in using siRNA as a potential therapeutic agent for many diseases (Lovett-Racke et al., 2005).

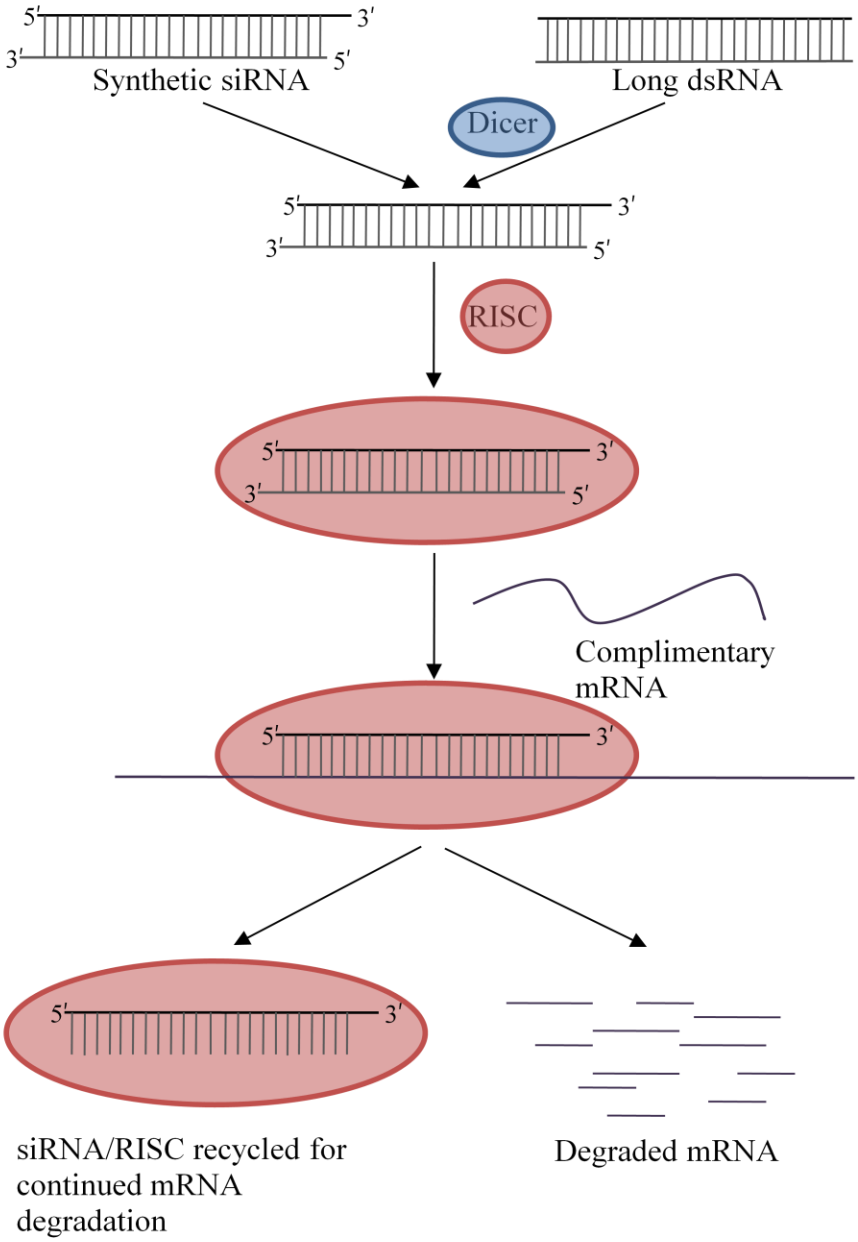


Figure 1.4. Mechanism of siRNA. SiRNAs are short nucleotide sequences that induce the degradation of complimentary mRNA sequences. DsRNA detected in the cell is cleaved by Dicer into 21 to 23 nucleotide siRNAs. These siRNA then bind to RISC, where the strands are separated. The single stranded RNA bound to RISC (the guide strand) complexes with complimentary mRNA. The mRNA is cleaved by an enzyme present in RISC, thereby preventing translation of the transcript. Once mRNA degradation is complete, the guide strand remains bound to RISC, allowing for the complex to bind with additional mRNA, thereby amplifying the degradation of the target mRNA. Synthetic siRNAs introduced into the cells can are potential therapeutic agents.

Recent Work in Modifying CS-GAG Chain Synthesis

An alternative approach to using chABC in removing inhibitory CS-GAGs from the injury site is to alter CSPG producing cells. In particular, astrocytes can be genetically engineered to secrete CSPGs without CS-GAGs or with modified CS-GAGs by knocking down the enzymes responsible for CS-GAG chain synthesis.

Xylosyl-Transferase-1

One approach that has been examined is to knockdown XT-1 in vivo by locally delivering a DNA enzyme which specifically degrades XT-1 mRNA (Grimpe and Silver, 2004), thereby preventing glycosylation in the cells that uptake the enzyme. This approach was successful at reducing CS-GAG intensity at the lesion penumbra, and neurites were able to regenerate around the injury site. However, because xylose is part of the tetrasaccharide common to *all* sulfated proteoglycans, not only CSPGs, this enzyme is not an ideal target. In particular, several HSPG (heparan sulfate proteoglycans) binding proteins have been implicated in the promotion of neurite outgrowth (Kinnunen et al., 1996), and, therefore, preventing HSPG chain polymerization is not favorable.

Chondroitin Polymerizing Factor

In a recent study, siRNA was used to knock down ChPF in an astrocytic cell line in vitro. Preventing CS-GAG chain polymerization was as effective as chABC in reducing the inhibitory properties of the conditioned astrocyte media (Laabs et al., 2007). Neurites from primary neurons grown on spots made from conditioned media of siRNA

treated cells were able to cross into the spot more readily than those grown on control spots. Although these results are promising, this approach, as with chABC, leaves behind an "inhibitory stub" on the CSPGs (Lemons et al., 2003).

Purpose of Thesis Project

The objective of this thesis was to explore the use of siRNA as an alternate therapy to chABC for promoting axonal regeneration after injury to the CNS. Sulfation affects both the physical and chemical characteristics of CS-GAGs and, therefore, it has been hypothesized that certain sulfation patterns may be more inhibitory than others. To investigate this hypothesis, specific CSSTs, the enzymes responsible for CS-GAG sulfation, were knocked down in vitro using siRNA. C4ST-1, C4ST-2, and C46ST were chosen as targets for gene knockdown in this study based on their expression in neural tissue and the extent of inhibition caused by their respective CS-GAG. C6ST-1 and and it's CS-GAG, CS-6, are both upregulated after injury (Properzi et al., 2005), though neither C6ST was chosen as a target for knockdown as CS-6 is the least inhibitory of all of the CS-GAGs (Gilbert et al., 2005). Although there are three known sulfotransferases responsible for CS-4 sulfation, only C4ST-1 and C4ST-2 are explored herein. In humans, C4ST-3 is expressed in the liver and kidney and is not found in neural tissue (Kang et al., 2001), whereas the expressions of C4ST-1 and C4ST-2 are more widespread (Hiraoka et al., 2000). This finding has been verified in mice (Akita et al., 2007). Therefore, it was hypothesized that transfection of primary rat astrocytes with siRNAs designed to prevent the expression of C4ST-1, C4ST-2, and C46ST would decrease specific sulfation patterns secreted by these cells. Conditioned culture media from the transfected astrocytes was

collected and used in neurite growth assays to determine the effect of sulfation on neurite extension.

Hypothesis

Successful treatment of the injured spinal cord must overcome the inhibitory effects of the glial scar while maintaining its beneficial properties. The **central hypothesis** of this thesis is that *specific CS-GAG sulfation patterns, in particular CS-4 and CS-4,6, dictate the inhibitory properties of CSPGs, and that modulation of sulfation patterns will promote neurite extension.*

Objectives

The overall objective of this thesis research is to determine if neurite inhibition can be alleviated through modulating CS-GAG sulfation. To test this hypothesis, the following objectives were set:

1. Knockdown CSSTs in astrocytes in vitro using siRNA
2. Determine the effect of siRNA knockdown on neurite outgrowth in vitro

CHAPTER 2

MATERIALS AND METHODS

Cell culture

Astrocyte Isolation and Culture

Primary cortical astrocytes were harvested from the brains of postnatal day 1 rat pups (Harlan). The pups were anesthetized with isoflurane (Henry Schein), decapitated, and the brain was removed. The cerebral cortices were microdissected out and the meninges were removed. The isolated cortical tissue was then minced and stored in Leibovitz's L-15 media (Invitogen). The tissue was digested by subsequent incubations with 0.25% collagenase (type 4) (Worthington Biochemical Corporation) and 0.25% trypsin and then dissociated by mechanically disrupting the digested tissue with a micropipette tip. Dissociated cells were plated in a T75 flask containing Dulbecco's Modified Eagle's Media/Ham's F-12 50/50 Mix (DMEM/F12; Mediatech) containing 10% fetal bovine serum (FBS; Hycolone) and 1% penicillin streptomycin (PS; MP Biomedicals). The astrocyte cultures were purified by mechanically removing neurons and microglia at 24 and 72 hours. Cultures were passaged at 90% confluency and maintained in T225 flasks. Unless otherwise noted, cells were used between passage 4 and passage 7 for experiments.

Cortical Neuron Isolation and Culture

Primary cortical neurons were harvested from the brains of rat fetuses (Embryonic day 18). Timed pregnant Sprague Dawley rats were anesthetized using isoflurane and

then rapidly decapitated. The uterus was removed by caesarean section, and the pups were removed from the amniotic sac into ice cold hank's balanced salt solution (HBSS) where they were then quickly decapitated. The brains were removed from the fetuses, freed from meninges and the cortical tissue was isolated by microdissection. The isolated tissue was digested with 0.25% trypsin EDTA for 10 minutes at 37^{o}C and then dissociated through trituration in the presence of 0.15mg/mL deoxyribonuclease I (DNase; Sigma) in HBSS. Viability and concentration of dissociated cells was determined using the trypan blue exclusion assay. For neurite outgrowth assays, cells were resuspended in Neurobasal containing B27 supplement (Invitrogen), 500µM l-glutamine (Hyclone), and 1% PS, and plated at 35,000 cells/cm^2.

Transfection of Astrocytes with siRNA

Quantification of Transfection Efficiency

The transfection efficiency of primary rat astrocytes with FuGENE®HD Transfection Agent (Roche), was assayed using siGLO® Green Transfection Indicator (Dharmacon). 24 hours prior to transfection, astrocytes were removed from tissue culture flasks by trypsinization and plated onto 6-well plates in complete growth medium. 3X10^5 cells were plated per well and 3 wells were plated per condition. Just prior to transfection, media was removed and replaced with serum free DMEM/F12. For each condition 300µL (100µL/well) of serum free media was added to a microcentrifuge tube. To these tubes siRNA (12µL total, 4µL/well) and FuGENE (12µL total, 4µL/well) were added and allowed to complex by incubating for 15 minutes at room temperature. 100µL of the media was then added to the appropriate well and the plates were placed in a 37^{o}C

incubator. After 48 hours, one set of the cells was again transfected using the same protocol. After 72 hours, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde, and stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), in order to visualize nuclei. A random image was taken of each well at 10X magnification with a Zeiss Axiovert 200M microscope using the DAPI and FITC filters. Images were overlaid in Adobe Photoshop. Transfection Efficiency was defined as the percentage of cells with green siRNA was colocalized with the nucleus.

Determination of Optimal siRNA Concentration

SiRNA is toxic to cells at high concentrations and ineffective at sufficiently silencing gene expression at low concentrations (Reynolds et al., 2004). The ensuing studies knockdown three genes simultaneously; therefore, in order to minimize total siRNA concentration a dosing study was performed to determine the lowest concentration of each CSST siRNA that is still effective for significant silencing. In order to maximize mRNA degradation, for each gene, a pool of four siRNA constructs complimentary to four separate locations on the mRNA strand was used. The siRNA against C4ST-1 was ON-TARGETplus SMARTpool siRNA (Dharmacon), which is a premade pool of the four siRNA sequences. For C4ST-2 and C46ST, four separate siGENOME siRNA (Dharmacon) sequences were purchased and later pooled. In total, four concentrations of the C4ST-1 siRNA were examined; 0, 40, 60, and 80nM, and five concentrations of the C4ST-2 and C46ST siRNA were examined; 0, 40, 60, and 80nM, and 100nM (0, 10, 15, 20, and 25nM of individual sequences). Cells were transfected (n=3) at both 24 and 48 hours after plating using the protocol as described above. After

72 hours, the cells were lysed and RNA was extracted using the RNeasy Mini Kit (Qiagen). Successful knockdown was defined at mRNA levels 20% or less of control.

Knockdown of C4ST-1, C4ST-2, and C46ST

The ability to down regulate the chondroitin-4 sulfotransferases C4ST-1, C4ST-2, and the chondroitin-4,6 sulfotransferase C46ST in vitro was assayed using siRNA specifically designed to the transcripts. 24 hours prior to transfection, astrocytes were removed from tissue culture flasks by trypsinization and plated onto 6-well plates in complete growth medium. $4X10^5$ cells were plated per well, three wells were plated per condition, and the experiment was run two times. Cells were transfected both 24 and 48 hours after plating using the protocol as described above. The amount of siRNA used for each condition was what was determined in the previous section as being the lowest concentration that results in sufficient knockdown (Table 2.1). 72 hours post transfection conditioned media (CM) was collected and stored at -80^oC for future analysis and RNA was extracted from the cells. 72 hours was chosen as the endpoint because by this time the effects of the siRNA are detected and the astrocytes should be able to secrete an adequate amount of CSPGs. Each sulfotransferase was assayed separately, as well as a combination of the chondroitin-4 sulfotransferases (C4ST-1, C4ST-2), and all three sulfotransferases (C4ST-1, C4ST-2, C46ST).

To explore the kinetics of CS-GAG sulfation an additional study was performed where the media was changed 24 hours post transfection and then collected 72 hours post transfection. The purpose of this study was to reduce the amount of secreted CS-GAGs that were produced before CSST protein levels were decreased. In this experiment there

were six conditions: Control (no media change), Control (media collected at 24 hours), Control (media change at 24 hours, media collected at 72 hours), Triple KD (no media change), Triple KD (media collected at 24 hours), and Triple KD (media change at 24 hours, media collected at 72 hours). For each condition $4X10^5$ cells were plated per well, three wells were plated per condition, and the experiment was run one time. The astrocytes used in this experiment were passage 12.

Table 2.1. SiRNA Doses. For each experimental condition the lowest concentration of siRNA that still effectively silenced gene expression was used.

Condition	siRNA/well				FuGENE/ well
	C4ST-1	C4ST-2	C46ST	Total	
Control	0nM	0nM	0nM	0nM	4μL
	0μL	0μL	0μL	0μL	
C4ST-1	60nM	0nM	0nM	60nM	4μL
	6μL	0μL	0μL	6μL	
C4ST-2	0nM	40nM (4x10nM)	0nM	40nM	4μL
	0μL	4μL (4x1μL)	0μL	4μL	
C46ST	0nM	0nM	40nM (4x10nM)	40nM	4μL
	0μL	0μL	4μL (4x1μL)	4μL	
C4ST-1 + C4ST-2	60nM	40nM (4x10nM)	0nM	100nM	4μL
	6μL	4μL (4x1μL)	0μL	10μL	
C4ST-1 + C4ST- + C46ST	60nM	40nM (4x10nM)	40nM (4x10nM)	140nM	4μL
	6μL	4μL (4x1μL)	4μL (4x1μL)	14μL	

Quantification of the Effects of siRNA Mediated Gene Silencing

Quantitative RT- PCR

Successful gene silencing was verified by reverse transcription quantitative polymerase chain reaction (qRT-PCR). 72 hours post-transfection, mRNA was extracted from the cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. The concentration and purity of extracted RNA was calculated by measuring the absorbance of the RNA at 260nm and 280nm using a spectrophotometer. mRNA (0.5μg) from each condition was reverse transcribed into cDNA using an iCycler thermocycler (Bio-Rad) and the iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. The cDNA products were stored at -20^{o}C or used immediately for qRT-PCR. cDNA for each gene of interest was amplified for use in a standard curve by RT-PCR using the thermocycler. qRT-PCR was carried out with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad). The following protocol was used: activation step, 3 min at 95^{o}C; three-step cycling (40 cycles): denaturation, 30 s at 95^{o}C, annealing, 30 s at 55^{o}C, extension, 1 min at 72^{o}C. A melt curve was subsequently performed to confirm that there was no primer dimer formation in the PCR products, which began at 55^{o}C and increased to 95^{o}C in 0.4^{o}C increments. A standard curve for each gene of interest was performed every time the gene was analyzed, and used to obtain the relative quantity of the targeted gene for all samples. The following primer sequences were used: C4ST-1, Forward ACCTGAAGTTCCCCACCTATG, Reverse TTGGCACTGAGTAGTTGAACATTA, C4ST-2 Forward AGGCAACAGCTCTATAAACTCT, Reverse

ATTCTCAAAGGACCATCTCAGT; C46ST Forward ATAACAATGCCTGGACCTTCTTCT, Reverse GATCCCTGAGCATGACAAGGAG.

Neurite Guidance Assays

To determine if modulating CS-GAG sulfation patterns is effective in alleviating CSPG-mediated neurite inhibition, neurite guidance assays were performed. CM collected from siRNA treated and control astrocytes was concentrated (30 fold) using a YM-30 centrifugal filter (Millipore). The total protein concentration of the unconcentrated CM from each experimental condition was determined by using Protein Assay Dye Reagent (Bio-Rad) and comparing to a standard curve generated with bovine serum albumin (BSA; Sigma). It was assumed that no protein was lost during concentrating and the final volume of concentrated product was brought up so that the total protein concentration in each sample was 1200μg/mL. The concentrated CM was mixed 50:50 (by volume) with 1mg/mL Texas Red labeled Dextran (Invitrogen) (final concentration of CM 600μg/mL and Texas Red 500μg/mL) for visualization purposes. A 3μL drop of the CM was spotted onto a 12mm poly-L-lysine (PLL) coated glass coverslip placed into the well of a 24 well plate, incubated at 37^{o}C in humidity for 2 hours, and then rinsed with sterile PBS. This created a distinct boundary between the PLL and the proteins in the CM that had adsorbed to the surface. Cortical neurons were seeded onto the coverslips (70,000/well, n=6) in Neurobasal media supplemented with B27 and l-glutamine. The cells were plated so that they covered the entire coverslip. After 48 hours, the cells were fixed using 4% paraformaldehyde and stained with anti-MAP2 antibody (1:500, Chemicon) at 4^{o}C overnight. For secondary detection the cells

were then incubated for 2 hours with Alexa Fluor® 488 goat anti-mouse IgG_1 (1:220, Molecular Probes). Montages at 20X magnification were taken with a Nikon Eclipse 80i microscope using Neurolucida software. Percent crossing was determined by dividing the number of neurites that cross over from PLL onto the spot by the total number of neurites within the distance of the size of one soma of the boundary.

To verify that CSPGs did adhere to the tissue culture plates, separate spots using control astrocyte media were made as described above. The coverslips were fixed with 4% paraformaldehyde and stained with CS-56 (Sigma), an antibody specific to chondroitin sulfate. The primary antibody (CS-56) was used at a 1:200 dilution and incubated at 4^{o}C overnight. The secondary antibody (Alexa Fluor® 488 goat anti-mouse IgM) was used at a 1:220 dilution and incubated 2 hours at room temperature. After staining, fluorescent images using both FITC and Texas Red filters were taken of the coverslips using a Zeiss 200M microscope. Images were overlaid using Adobe Photoshop to show colocalization of Texas Red and CS-56.

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) was performed on conditioned astrocyte media to determine if knocking down CSST expression has an effect on the CS-GAG content of proteins secreted by these cells. The total protein concentration of the CM from each experimental condition was determined by using Bio-Rad Protein Assay Dye Reagent and comparing to a standard curve generated using BSA. The volume of unconcentrated CM containing 1 μg of total protein was brought up to 200μL so that each sample was at a concentration of 5μg/mL. 50μL of these samples was added to the

wells (n=3 for each media sample) of a nitrocellulose-coated 96-well assay plates in triplicate. Nitrocellulose-coated plates were made by adding 20μL of a nitrocellulose/methanol solution (5cm^2 nitrocellulose (Schleicher and Schull) in 12mL methanol (Fisher)) to each well and drying the solution in a laminar flow hood. The proteins in the CM were allowed to adsorb to the assay plate for 6 hours at room temperature. After incubation and removal of the excess CM, nonspecific background was blocked by adding 200μl of blocking solution (2% powdered milk in TBST) for 1 hour at room temperature. CS-56 (1:1000) was then added and incubated overnight at 4^{o}C. Wells were washed 6 times with TBST and incubated with HRP-conjugated goat anti-mouse IgM (1:1000, Jackson Immunochemicals) for 1.5 hours. Next, the wells were washed 6 times with TBST and then incubated with 200μL of substrate solution (200μL of 15mg/mL 2,2'-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS, Southern Biotech) and 10μL of 30% H_2O_2 in 10mL 50mM citrate monohydrate (Sigma)) at room temperature for 30 minutes and the absorbance 410 nm was recorded. ChABC treated CM from control astrocytes were used at a positive control and serum free media was used as a negative control.

CHAPTER 3

RESULTS

Optimization of Conditions for CSST siRNA Transfection

siRNA Transfection Efficiency

The efficiency in which the FuGENE HD/siRNA complex is transfected into primary rat astrocytes was assayed by transfecting the cells with an siRNA sequence chemically modified with a green fluorescent dye. 72 hours post-transfection, the cells were fixed with 4% paraformaldehyde and stained with DAPI. Fluorescent images were taken at a random field in each well (n=3wells/condition) at 10X magnification using DAPI and FITC filters and the images were overlaid in Adobe Photoshop. Transfection Efficiency was determined by counting the number of nuclei colocalized with green dots, indicative of siRNA in the cells (Figure 3.1). The conditions assayed were Control Cells (no siRNA, no FuGENE), FuGENE Control (no siRNA), siRNA Control (no FuGENE), Single Transfection (transfected at t=0), and Double Transfection (transfected at t=0 and t=48 hours). For both the Control Cell and FuGENE Control conditions, 0% of the cells contained siRNA, confirming that the positive green signal was not due to autofluorescence. For siRNA Control only 1% (± 1%) of the cells contained siRNA. This demonstrates that a transfection agent is necessary for adequate uptake. 84% (± 17%) of the Single Transfection cells were positive for siRNA after 72 hours whereas 99% (±2%) of the Double Transfection cells contained siRNA. For this reason, it was decided to perform a double transfection for the remainder of the experiments.

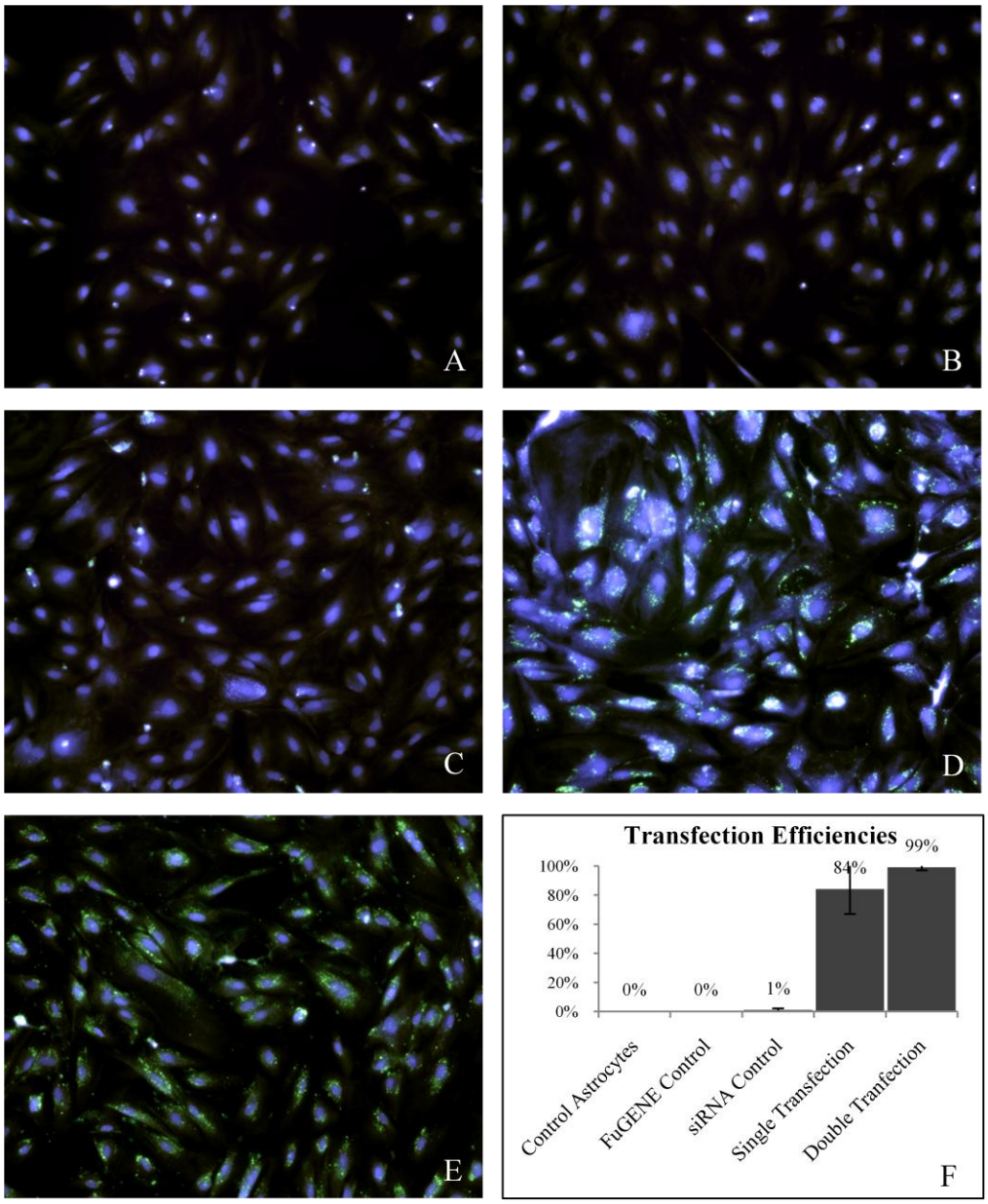


Figure 3.1. Transfection Efficiency of Primary Rat Astrocytes. Transfection efficiency was calculated by dividing the number of nuclei colocalized with the green fluorescent siRNA by the total number of nuclei. A) Control Cells (no siRNA, no FuGENE), B) FuGENE Control (no siRNA), C) siRNA Control (no FuGENE), D) Single Transfection (transfected at t=0), E) Double Transfection (transfected at t=0 and t=48 hours), F) calculated transfection efficiencies for all conditions.

Optimal Concentrations of C4ST-1, C4ST-2, and C46ST siRNA

SiRNA is cytotoxic at high concentrations but ineffective at sufficiently knocking down gene expression at low concentrations. Because subsequent studies include the simultaneous knockdown of three genes, a dosing study was performed to determine the lowest concentration of each CSST siRNA that is effective for significant gene silencing so that total siRNA concentration could be minimized. In total, four concentrations of the C4ST-1 siRNA were examined; 0nM, 40nM, 60nM, and 80nM, and five concentrations of the C4ST-2 and C46ST siRNA were examined; 0nM, 40nM, 60nM, 80nM, and 100nM. For all conditions, astrocytes were transfected at t=0 and t=48 hours. After 72 hours the cells were lysed, RNA was extracted, and qRT-PCR was performed to determine mRNA levels of the CSST being knocked down (Figure 3.2). To verify that the observed knockdown was specific to the gene of interest, qRT-PCR was also carried out for C4ST-2 in the C4ST-1 knockdown, C4ST-1 in the C4ST-2 knockdown, and C4ST-1 in the C46ST knockdown. Successful knockdown was defined as mRNA levels 20% or less of control. Each PCR reaction was run in triplicate and significance from control was determined using the Student's T-test. In the C4ST-2 and C46ST knockdowns all conditions were significant from control ($p < 0.05$). In the C4ST-1 knockdowns, the 40nM dose was not significant from control ($p > 0.05$) but 60nM and 80nM were ($p < 0.05$).

C4ST-1 siRNA was most effective with the 80nM dose though successful knockdown was also present with the 60nM dose. In order to minimize total siRNA concentration in the triple knockdown, it was decided to use the C4ST-1 siRNA at 60nM in subsequent studies. For both C4ST-2 and C46ST, the siRNA significantly knocked

down gene expression at the lowest concentration assayed (40nM). Therefore in subsequent studies it was decided to use these siRNAs at 40nM.

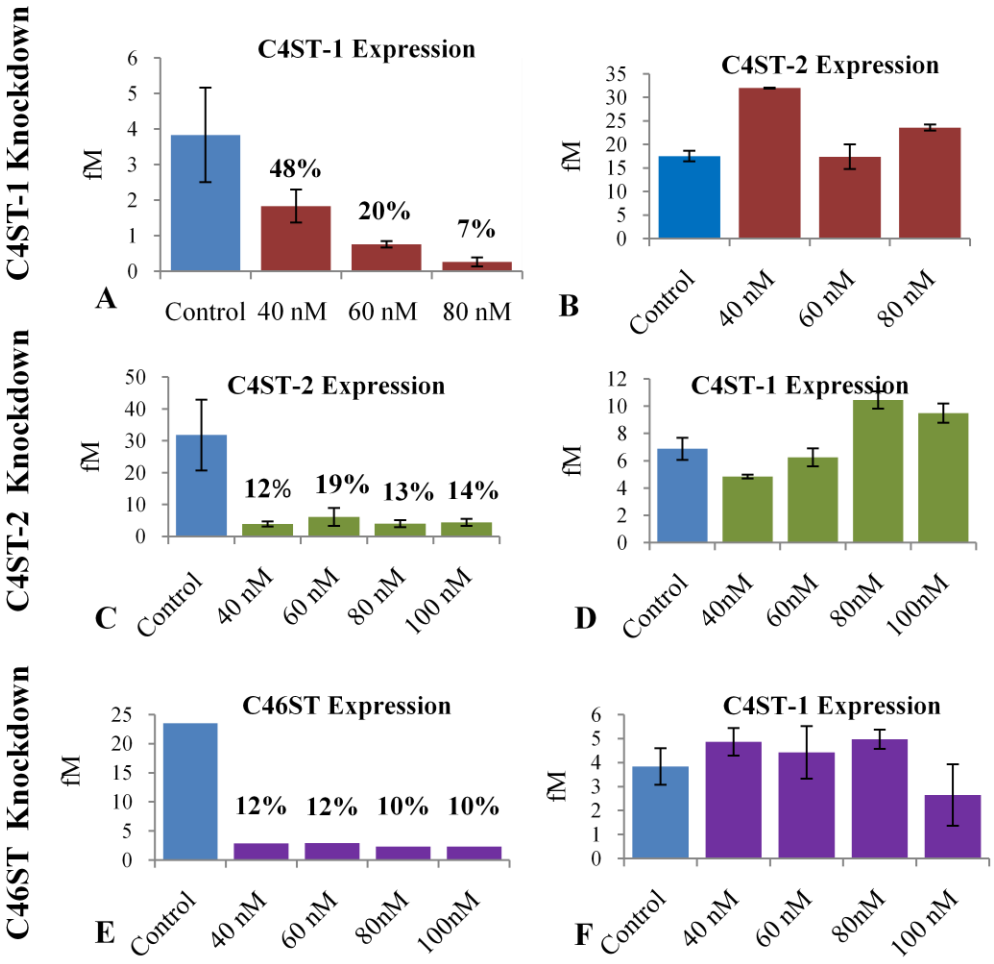


Figure 3.2. SiRNA Dosing. The effectiveness of siRNA at different concentrations was determined for C4ST-1, C4ST-2, and C46ST siRNA using qRT-PCR. Successful knockdown was defined as mRNA levels 20% the control or lower. A) C4ST-1 mRNA expression after transfection with C4ST-1 siRNA. B) C4ST-2 mRNA expression after transfection with C4ST-1 siRNA. C) C4ST-2 mRNA expression after transfection with C4ST-2 siRNA. D) C4ST-1 mRNA expression after transfection with C4ST-2 siRNA. E) C46ST mRNA expression after transfection with C46ST siRNA. F) C4ST-2 mRNA expression after transfection with C46ST siRNA.

Functional Outcomes of CSST Knockdown Experiment

CSST mRNA is Down-Regulated with CSST Specific siRNA

Previous work has shown that CS-GAGs are inhibitory to neurite outgrowth, even in the absence of a protein core, and that the dual sulfated CS-GAG, CS-4,6, is the most inhibitory CS-GAG followed by CS-4 (Gilbert et al., 2005). To establish if CS-GAG sulfation patterns can be modulated by silencing the expression of specific CSSTs, and if this modification is sufficient in suppressing CSPG mediated inhibition of neurite outgrowth, the genes for three CSSTs were knocked down in primary rat astrocytes in vitro using siRNA designed to the target mRNA. Specifically, the chondroitin-4 sulfotransferases C4ST1, C4ST2 and the chondroitin-4,6 sulfotransferase C46ST were chosen as targets since these enzymes are responsible for sulfating the most inhibitory of the CS-GAGs.

For this study, each of the three sulfotransferases was knocked down separately, as well as a combination of the chondroitin-4 sulfotransferases (C4ST-1, C4ST-2), and all three sulfotransferases (C4ST-1, C4ST-2, C46ST). Successful knockdown was verified by qRT-PCR. In addition qRT-PCR for all three genes was performed on all conditions to verify that the observed decrease mRNA was specific to the targeted mRNA (Figure 3.3). Each PCR reaction was run in triplicate and significance from control was determined using the Student's T-test. For all conditions the mRNA levels for the CSSTs corresponding to the siRNA treatment were significantly different than control ($p<0.05$) and <15% of the control. The mRNA for the other CSSTs were at levels comparable to

the control, indicating successful specific knockdown (only C4ST-1 mRNA levels in the C4ST-2were significantly different than control).

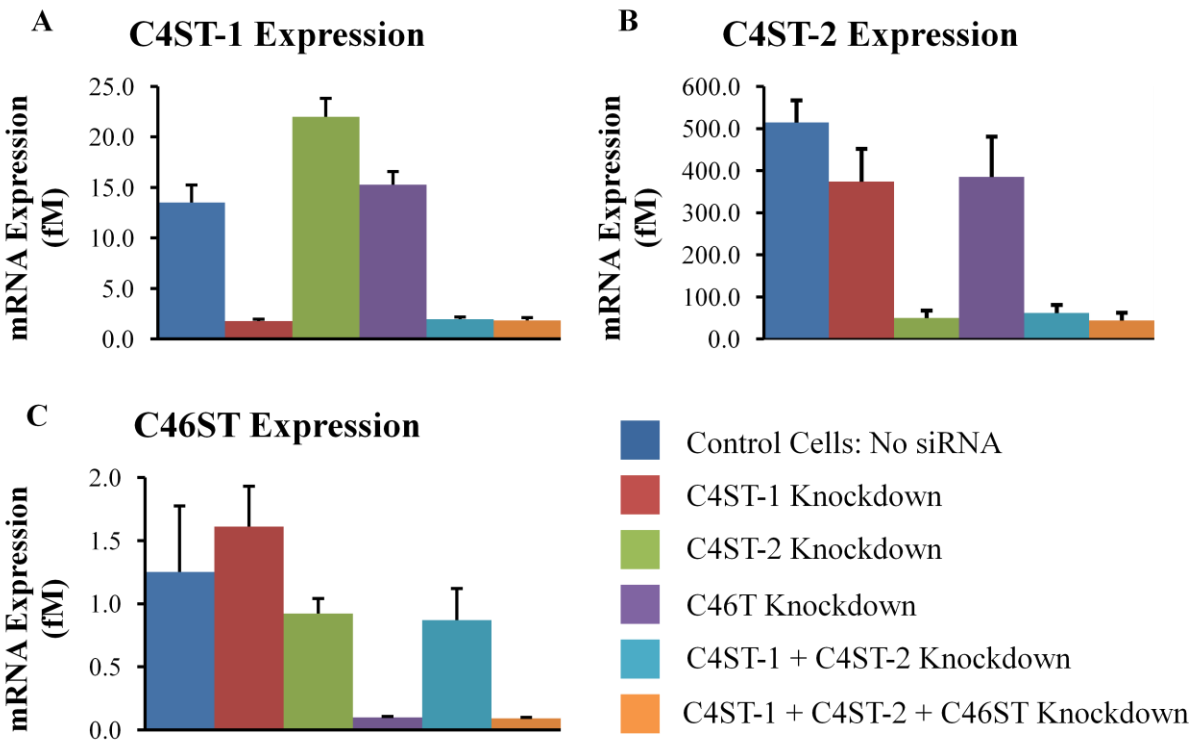


Figure 3.3. CSST mRNA Expression. A) C4ST-1 mRNA is specifically silenced in the three conditions receiving C4ST-1 siRNA. B) C4ST-2 mRNA is specifically silenced in the three conditions receiving C4ST-2 siRNA. C) C46ST mRNA is specifically silenced in the two conditions receiving C46ST siRNA. For all samples where silencing is observed the mRNA levels are <15% of the control mRNA levels.

ELISA for Quantification of Total CS-GAG Content

In the previous section it was verified by qRT-PCR that the gene expressions of the CSSTs are being silenced; however, the absence of an antibody specific to the targeted CSSTs makes it difficult to verify successful knockdown on the protein level. Nevertheless, reduction in the total amount of GAGs secreted by the astrocytes could be measured using a CS-56 ELISA, which specifically measures CS-GAGs.

In the first set of experiments, all CM was collected 72 hours after the first transfection. A CM sample treated with chABC was used at a positive control. Significance from control was determined using the Student's T-test. Results show a slight decrease in CS-GAG sulfation for all siRNA treated samples; however, the only condition that was significantly different from the control was the triple knockdown (Figure 3.4).

Given that it takes about 24 hours for significant gene knockdown to occur, it is conceivable that CS-GAGs produced in the first 24 hours dominate, or effectively dilute, CS-GAGs produced later. To address this possibility, the astrocyte media was changed 24 hours after transfection and then collected 48 hours later. Total CS-GAG content in this media was compared to those in media that was not changed and collected after 72 hours (Figure 3.5). The results of this experiment showed that after 72 hours, the CS-56 concentration in CM from the siRNA treated astrocytes (only triple knockdown was assayed) was about 60% that of control astrocytes ($p<0.05$), whereas when there was no media change there was no difference in the CS-56 intensity between the groups ($p>0.05$). These results are contradictory to the previous experiment, where in the absence of a media change the CS-56 intensity in the triple knockdown was significantly

less than the control cells. The astrocytes used in this experiment were of a much later passage (P = 12 compared to P = 4-6) and, since PCR was not done to verify knockdown, it is conceivable that transfection efficiency, and hence the level of protein knockdown, was lower in this study.

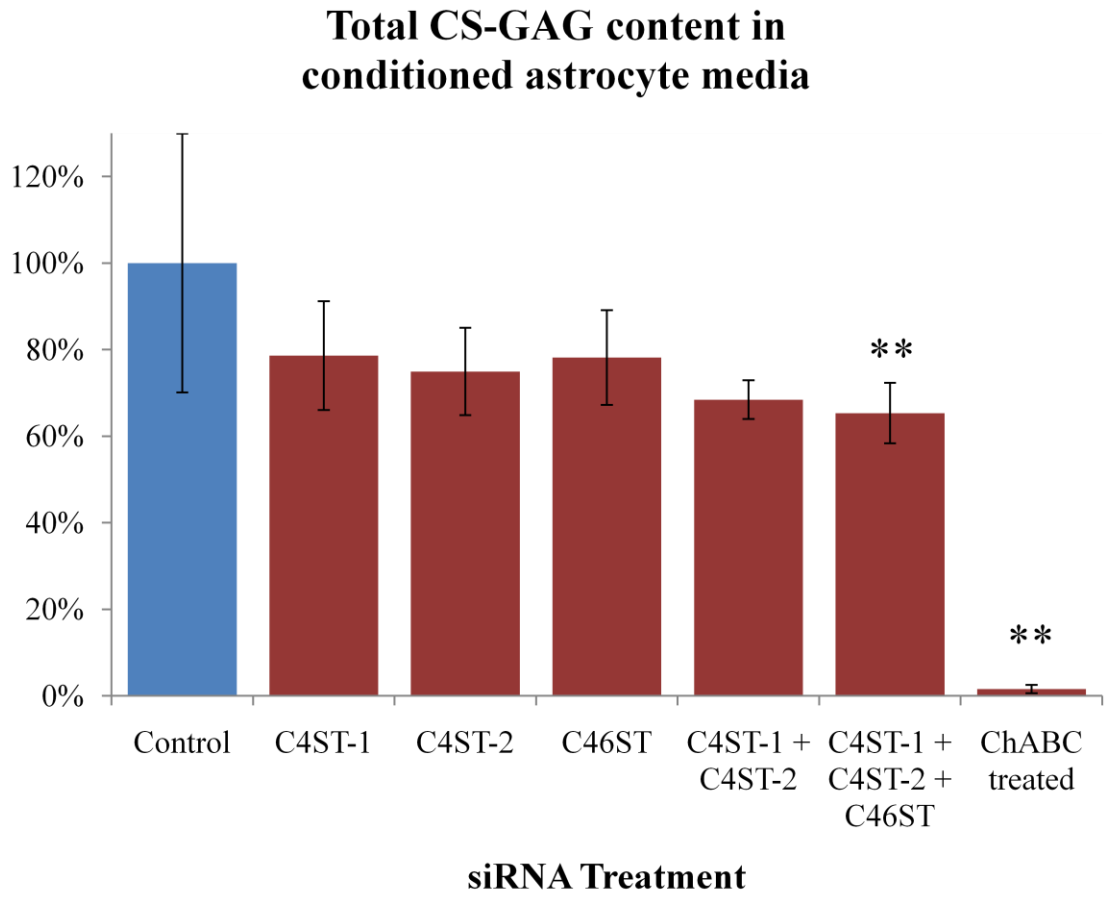


Figure 3.4 CS-GAG ELISA I. The total amount of CS-GAGs in the CM was determined by performing a CS-56 ELISA on the CM samples. ** indicates significant difference to control ($p < 0.05$).

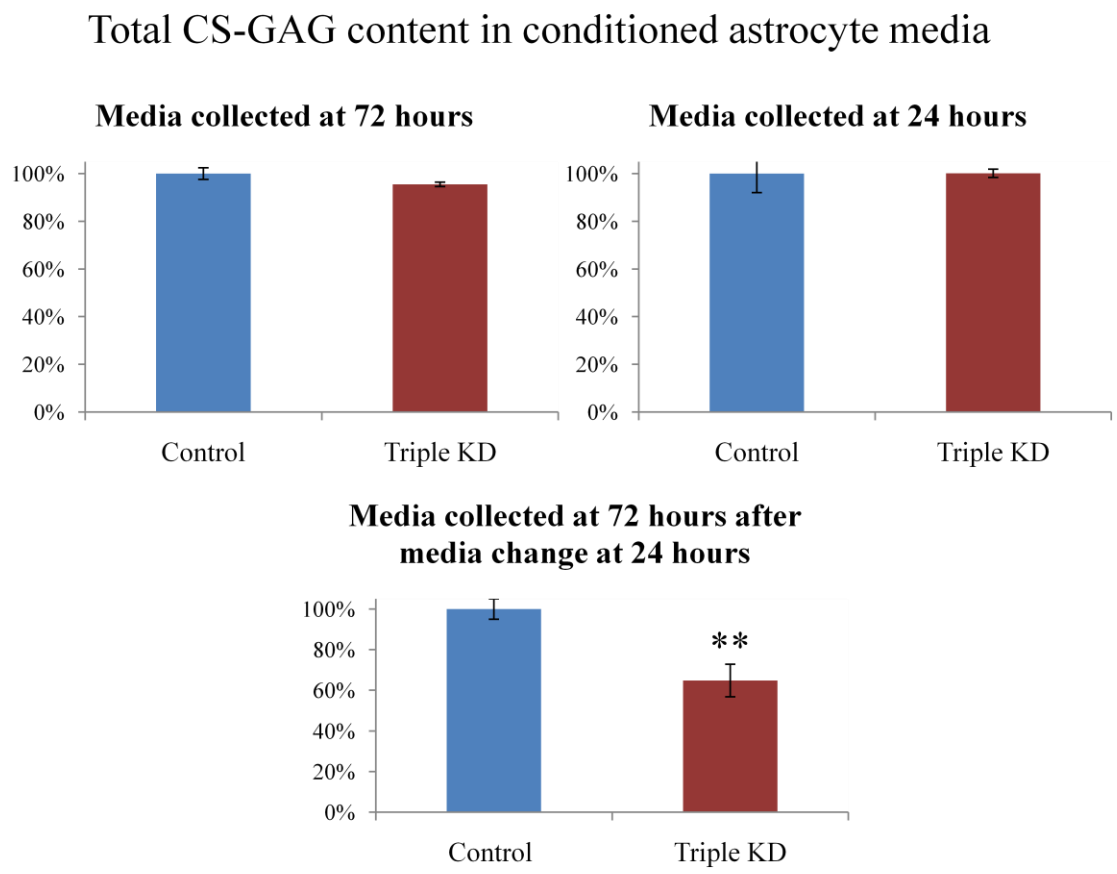


Figure 3.5. CS-GAG ELISA II. Media was changed after 24 hours and the total amount of CS-GAGs in the CM when was determined by CS-56 ELISA. In this experiment there were six conditions: Control (no media change), Control (media collected at 24 hours), Control (media change at 24 hours, media collected at 72 hours), Triple KD (no media change), Triple KD (media collected at 24 hours), and Triple KD (media change at 24 hours, media collected at 72 hours). ** indicates significance from control.

Neurite Guidance Spot Assays

It has been hypothesized that specific CS-GAGs contribute more to CSPG-mediated inhibition of neurite outgrowth, and, therefore, preventing expression of these CS-GAGs by knocking down their respective CSSTs will alleviate the inhibitory properties of conditioned astrocyte media. To test this hypothesis, a neurite guidance spot assay was performed. CM collected from control and siRNA treated astrocytes (from experiment where media was NOT changed at 24 hours) was concentrated (to a final total protein concentration of 1.2mg/mL) and spotted onto PLL coated glass coverslips, creating an interface between PLL and the proteins found within the conditioned media, including many CSPGs. Texas Red was mixed with the CM in order to visualize the PLL/protein boundary. Primary cortical neurons were cultured on the coverslips for 48 hours and fixed and stained with anti-MAP2 antibody. Montages at 20X magnification were taken for image analysis. To verify that CSPGs from the conditioned media did adhere to the coverslip and that Texas Red was sufficient in representing the boundary, separate spots using control astrocyte CM were made. The coverslips were fixed with 4% paraformaldehyde and stained with CS-56, an antibody specific to CS-GAGs. Fluorescent images at 10X magnification were taken of the spot using both Texas Red and FITC filters. To visualize the colocalization of Texas Red and CS-56 images were overlaid in Adobe Photoshop (Figure 3.6). There was a strong CS-56 signal throughout the spot, especially along the edge, and CS-56 staining colocalized with Texas Red, demonstrated that the boundary delineated by Texas Red is adequate to use in the neurite outgrowth assays.

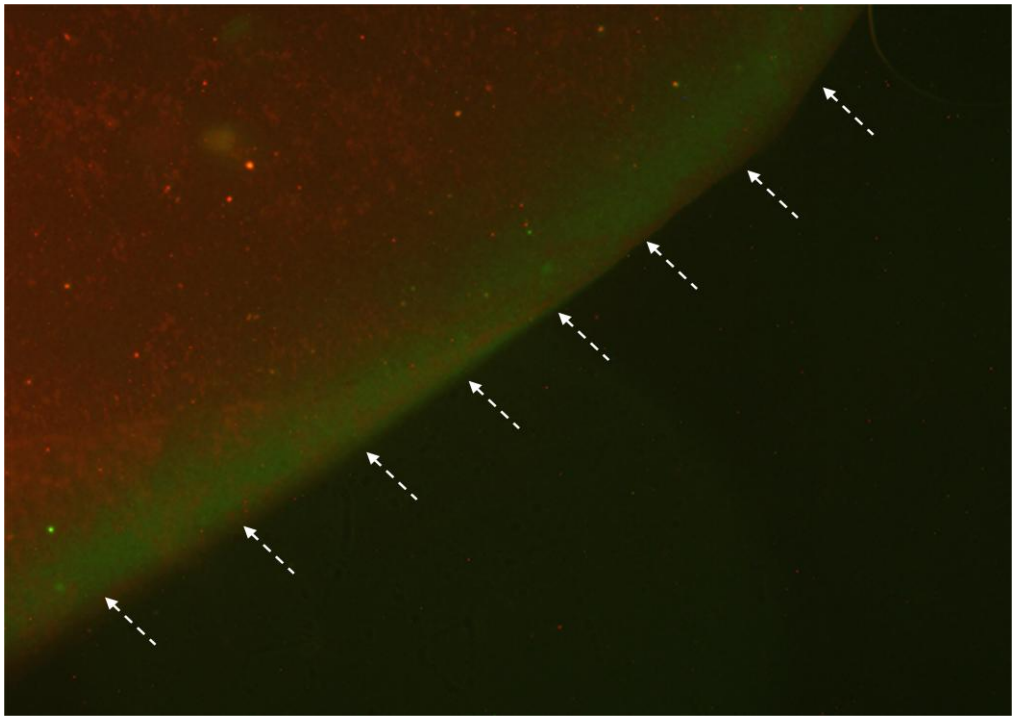


Figure 3.6. Colocalization of CS-56 and Texas Read. To confirm that the spots contained CS-GAGs, and that the Texas Red boundary is the same as the CS-GAG boundary spots using control astrocyte media stained with CS-56. Overlaid images show that Texas Red and CS-56 staining are colocalized, ensuring that the Texas Red boundary is adequate to use in outgrowth assays.

Percent crossing was determined by dividing the number of neurites that cross over onto the spot by the total number of neurites within the distance of the size of one soma of the boundary. Figure 3.7 demonstrates representative images of a neurite not crossing (Figure 3.7A) and a neurite crossing (Figure 3.7B). Six coverslips were assayed per condition and significance from control was determined using the Student's T-test. For neurons grown on the spots made of control CM, 43±3% of the neurites crossed from the PLL into the spot (Figure 3.8) and there was no significant difference between any of the groups.

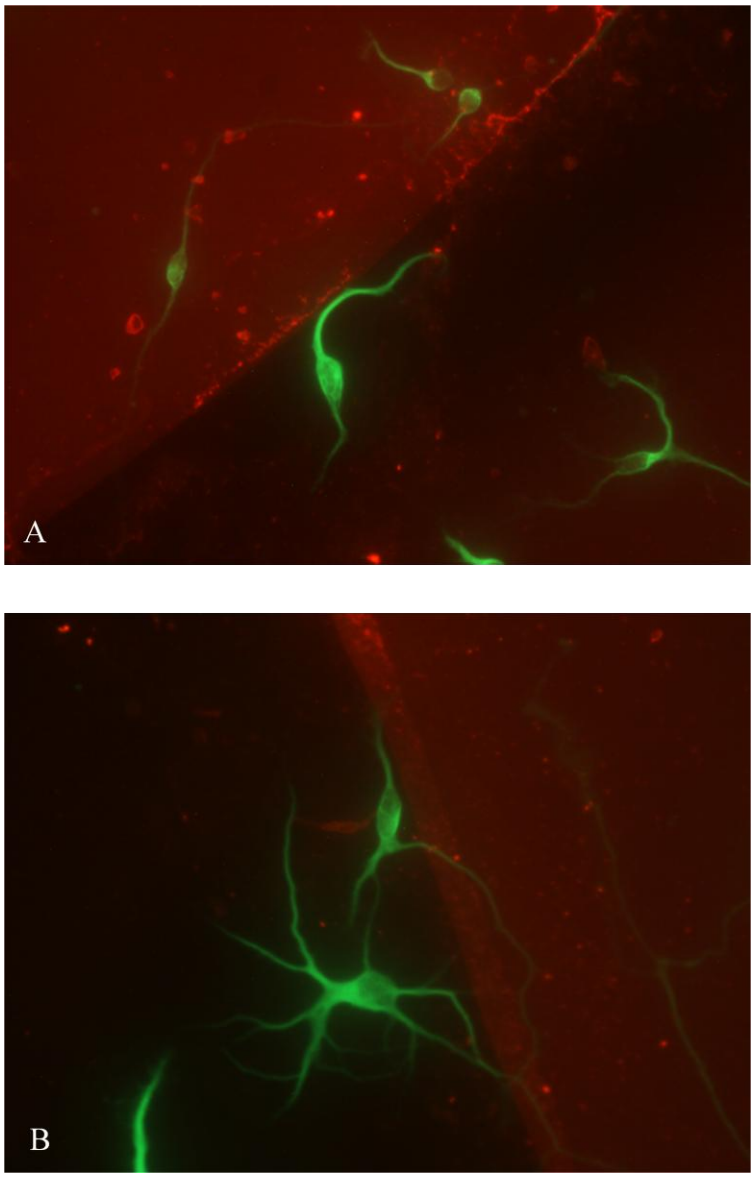


Figure 3.7. Neurite Guidance Spot Assays: Representative Images. MAP-2 stained neurons (green) grown on coverslips spotted with conditioned astrocyte media. Texas Red was added to the media for visualization purposes (red). Neurites A) not crossing and B) crossing

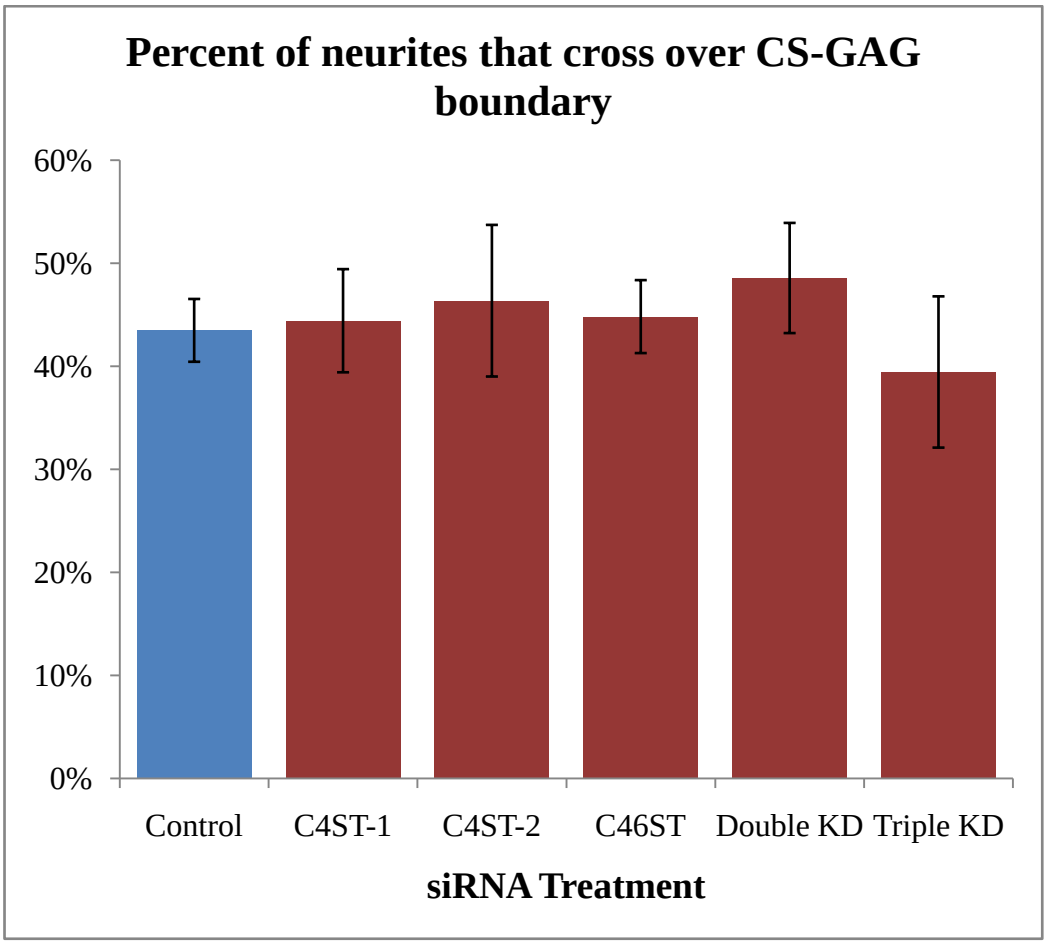


Figure 3.8. Neurite Guidance Spot Assays: Quantification of Crossing. Percent crossing was determined by dividing the number of neurites that cross over onto the spot by the total number of neurites within the distance of the size of one soma of the boundary. There was no significant difference between any of the groups.

CHAPTER 4

DISCUSSION

It is evident that CSPGs and CS-GAGs play a role in the regenerative failure of the CNS, though the exact mechanism through which this inhibition occurs remains unclear. Previous work has shown that CS-GAGs inhibit neurite outgrowth in the absence of a core protein, and that the degree of inhibition is dependent on the position and extent of sulfation, with the dual sulfated CS-GAG, CS-46, being the most inhibitory followed by CS-4 (Gilbert et al., 2005). Furthermore, treatment with sodium chlorate, which prevents CS-GAG sulfation, alleviates the inhibitory properties of CSPGs (Smith-Thomas et al., 1995). With such evidence implicating that sulfation may be at least partly responsible for regenerative failure in the CNS, we hypothesized that neurite outgrowth could be promoted through modulating the sulfation patterns of CSPGs secreted by astrocytes. To test this hypothesis, we knocked down various CSSTs, the enzymes responsible for CS-GAG sulfation, in primary rat astrocytes with the intention of controlling the sulfation profile of the CSPGs produced by these cells. Specifically, we chose to target the three enzymes responsible for sulfating the two most inhibitory CS-GAGs to CNS regeneration: the chondroitin 4,6- sulfotransferase, C46ST, and the chondroitin 4-sulfotransferases C4ST-1 and C4ST-2.

The ability to increase the regenerative potential of CNS neurons through the modification of sulfation patterns in the glial scar has important clinical implications. The formation of the glial scar, consisting mainly of CSPGs, is the primary cause of regenerative failure in CNS regeneration. However, preventing glial scar formation,

either by inhibiting the proliferation of reactive astrocytes (Myer et al., 2006) or administering anti-TGFβ-1 antibodies (King et al., 2004), causes further secondary damage and, in turn, results in a greater lesion volume. Therefore, an effective therapy must overcome inhibition caused by the glial scar without disrupting its integrity and, consequently, its favorable properties. In principle, this may be accomplished by controlling sulfation content.

Although targeting the CS-GAG chain biosynthetic pathway may be successful in controlling the inhibitory properties of the glial scar, there are several issues that could arise with this approach. One concern is that the targeted enzyme may have multiple activities and knocking down the protein, and in turn its other functions, may result in undesirable side effects. In principle, this should not be a problem with the enzymes explored herein, as the only known role of CSSTs is their chondroitin sulfating function (www.ncbi.nlm.nih.gov/sites/entrez?db=gene, 2008). A second concern is that more than one isoform of the protein may exist which may compensate for the knocked down protein. Given that there are seven known CSSTs, three of which are 4-sulfating, it is possible that this problem could arise. Not only may one isoform compensate for another with the same sulfating function, another type of CSST may compensate causing an increase in an alternate sulfation pattern. Knocking down all seven enzymes would be very difficult because the siRNA dose necessary to successfully accomplish this is most likely cytotoxic. Assuming that the second scenario does not occur, significant improvement in CNS regeneration might be achieved by targeting only two of the seven CSSTs. Even though there are three isoforms of C4ST, only two are present in the CNS, and because CS-46 is produced by adding a 6-sulfate to an already present CS-4, the

overall levels of CS4,6 and CS-4 should be concomitantly reduced by knocking down C4ST-1 and C4ST-2.

SiRNA are appealing candidates as therapeutic agents given their high specificity and ability to control gene function using a naturally occurring cellular pathway. There are, however, potential downsides to this approach. For instance, when targeting a multicellular environment, such as the CNS, it is difficult to ensure transfection of the appropriate cell type. Ubiquitous cellular uptake of siRNA would not only decrease the knockdown efficiency in target cells, but could also potentially cause detrimental side effects in other cells. Cellular targeting could be accomplished by modifying the siRNA or the delivery vehicle with a ligand to a cell specific receptor (McNamara et al., 2006). Additionally, the duration of knockdown must be taken into consideration when using siRNA. Although one strand of siRNA is capable of degrading many strands of mRNA, its effects are still transient. In vitro it has been shown that with a single dose of siRNA, mRNA levels return to control levels 96 hours after transfection (Laabs et al., 2007). Appropriate duration may be accomplished using a slow release delivery system, such as degradable polymeric microparticles or lipid microtubules (Khan et al., 2004). Furthermore, the mode of siRNA delivery may provoke an immune reaction, causing further damage.

Unfortunately, the in vitro neurite guidance assay demonstrated no decrease in inhibition caused by CM from siRNA treated astrocytes compared to CM from control astrocytes. Although these results contradict our hypothesis, it cannot be unambiguously concluded that modulating sulfation does not affect the inhibitory properties of glial scar. There are several explanations for why the expected decrease in inhibition was not

observed in our studies. First, while spot assays using non-conditioned, serum free media had significantly more crossing than the Control Condition assay (data not shown), the difference was not substantial (<10% difference), potentially indicating that an insufficient amount of CSPGs were produced by control astrocytes. Stimulating the astrocytes with TGFβ has been shown to significantly increase CSPG production in astrocytes (Smith and Strunz, 2005), which in turn should increase the inhibition caused by the CM. Alternatively, it is possible that a sufficient amount of CSPGs was present in the media, but there was not a significant difference in the CS-GAG content in the various media samples because the media was not collected at the appropriate time point. That is, the concentration of the CSPGs produced before the siRNA took effect may be greater than the concentration of the CSPGs produced after. Moreover, it is also conceivable that neurons used were not appropriate for the spot assay. E18 cortical neurons were chosen as the model system because the dissection and culture protocol has been well established in the lab, and previous findings indicate that the length of neurites from these cells grown on CSPGs is shorter than cells grown on control substrates (Dergham, 2002). However, the regenerative capacity of these cells may be too great, and the assay may be more effective using an alternative cell types, such as post-natal cerebellar granule neurons or hippocampal neurons.

CHAPTER 5

FUTURE DIRECTIONS

Optimization of Collection of Conditioned Media after Transfection

To fully understand the importance of sulfation in CSPG-mediated neurite outgrowth, there are several adjustments to the experiments that can be made. Most importantly, the optimal time point of media collection after siRNA delivery must be determined. It is possible that no difference in neurite outgrowth between the experimental groups in the spot assay was observed because there was no significant difference in the sulfation content of the media. That is, the CSPGs produced before CSST silencing may be overpowering the CSPGs produced thereafter. SiRNA induced gene knockdown can be detected 24 hours after transfection, and possibly even earlier. However, it is uncertain how gene knockdown correlates to a decrease in CSST protein expression and in turn CS-GAG sulfation. To characterize the kinetics of CSST knockdown, sulfation profiles of CM from various time points can be determined using HPLC (details explained below).

Cytokine Stimulation

Previously, it has been shown that CSPG production is upregulated in astrocytes in vitro when they are stimulated with various cytokines (Smith and Strunz, 2005). One explanation to why there is no significant difference in the inhibition to neurite outgrowth caused by the CM of the siRNA treated cells compared to that of control CM is that the astrocytes are not secreting adequate levels of CSPGs and therefore the CM from control

astrocytes is not significantly inhibitory. Therefore, increasing CSPG production through stimulating the astrocytes to promote CSPG secretion may increase the inhibition caused by the control CM. Additionally, the presence of cytokines will closer mimic the environment present after injury.

HPLC to Determine Sulfation Profile

Although an ELISA is capable of detecting changes in the total amount of CS-GAGs secreted by astrocytes, it cannot differentiate between the various sulfated GAGs, and, therefore, is unable to demonstrate which CS-GAGs are contributing to the total content. In future studies we would like to complete a sulfation profile of the CM to ensure that there is a decrease in expression of CS-GAGs corresponding to the CSSTs that are being silenced. Additionally, the possibility that there is an increase in the concentration of other sulfated GAGs to compensate for the CS-GAGs that are being suppressed must be explored. This profiling can be achieved using high performance liquid chromatography (HPLC).

Addition of Controls in Neurite Guidance Spot Assay

To verify that the neurite guidance spot assay is effective in assessing how inhibitory the CM is, further controls must be included. One necessary positive control is chABC treated CM from control astrocytes. Previously it has been shown that neurons grown in the presence of chABC treated astrocytic CM exhibit improved neurite outgrowth (Laabs et al., 2007) and therefore the same outcome is expected to occur in this experimental system. An additional positive control is CM from astrocytes treated

with chlorate, a chemical that inhibits ATP sulphurylase, thereby reducing CS-GAG sulfation (Hoogewerf et al., 1991). This control will show the effects of non-sulfated CSPGs on neurite outgrowth. A negative control that must be included is spots made from a known concentration of aggrecan or another intact CSPG. This control will verify that the spots are effectively inhibiting neurite outgrowth.

Delivery of siRNA in an Injury Model

Once we demonstrate that modulation of CS-GAG sulfation decreases CSPG-mediated inhibition of neurite outgrowth in vitro, the feasibility of using this approach in vivo will be explored. More specifically, CSST siRNA will be delivered to a spinal cord injury model. Given that the glial scar takes several weeks to fully form (Fawcett and Asher, 1999), the siRNA will be slowly released into the area of injury over this time course using lipid microtubules or polymeric microparticles (Khan et al., 2004). Prior to in vivo delivery of the siRNA, in vitro release assays will be performed to determine the appropriate loading concentration of the vehicles as well as the number of vehicles to administer. Astrocytes will be transfected with the released siRNA to verify that it is effective after release. Once this is determined the siRNA will be administered in vivo. Neurite outgrowth into the glial scar will be examined through histological analysis along with functional recovery through behavioral tests.

A potential issue that may arise with the administration of siRNA in vivo is that the CNS is a multicellular environment and therefore it may be difficult to ensure that only the astrocytes are being transfected. Another possible complication is that transfection efficiency may be low without a transfection reagent. Both issues may be

solved through modifying the siRNA or the delivery vehicle with a ligand to a cell specific receptor (McNamara et al., 2006). This should increase both cell specificity and cellular uptake.

Determine How Effective C4ST siRNA is in Reducing CS-4,6 Levels

To minimize the total concentration of siRNA delivered to the cells, the feasibility of using only C4ST siRNA will be explored. Significant improvement in CNS regeneration may be achieved through targeting only C4ST-1 and C4ST-2, the two C4STs present in the CNS. CS-46 is produced by adding a 6-sulfate to an already present CS-4, therefore it may be possible to concomitantly reduce expression of CS-4 and CS-4,6 by knocking down C4ST-1 and C4ST-2.

REFERENCES

Aagaard L, Rossi JJ (2007) RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 59:75-86.

Akita K, von Holst A, Furukawa Y, Mikami T, Sugahara K, Faissner A (2007) Expression of Multiple Chondroitin/Dermatan Sulfotransferases in the Neurogenic Regions of the Embryonic and Adult CNS imply that complex Chondroitin Sulfates have a Role in Neural Stem Cell Maintenance. Stem Cells.

Almeida R, Levery SB, Mandel U, Kresse H, Schwientek T, Bennett EP, Clausen H (1999) Cloning and expression of a proteoglycan UDP-galactose:beta-xylose beta1,4-galactosyltransferase I. A seventh member of the human beta4-galactosyltransferase gene family. J Biol Chem 274:26165-26171.

Bai X, Zhou D, Brown JR, Crawford BE, Hennet T, Esko JD (2001) Biosynthesis of the linkage region of glycosaminoglycans: cloning and activity of galactosyltransferase II, the sixth member of the beta 1,3-galactosyltransferase family (beta 3GalT6). J Biol Chem 276:48189-48195.

Bartsch U, Bandtlow CE, Schnell L, Bartsch S, Spillmann AA, Rubin BP, Hillenbrand R, Montag D, Schwab ME, Schachner M (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. Neuron 15:1375-1381.

Bayliss MT, Osborne D, Woodhouse S, Davidson C (1999) Sulfation of chondroitin sulfate in human articular cartilage. The effect of age, topographical position, and zone of cartilage on tissue composition. J Biol Chem 274:15892-15900.

Bechard D, Gentina T, Delehedde M, Scherpereel A, Lyon M, Aumercier M, Vazeux R, Richet C, Degand P, Jude B, Janin A, Fernig DG, Tonnel AB, Lassalle P (2001) Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. J Biol Chem 276:48341-48349.

Becker CG, Becker T (2002) Repellent guidance of regenerating optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci 22:842-853.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636-640.

Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378:498-501.

Brittis PA, Silver J (1994) Exogenous glycosaminoglycans induce complete inversion of retinal ganglion cell bodies and their axons within the retinal neuroepithelium. Proc Natl Acad Sci U S A 91:7539-7542.

Brittis PA, Canning DR, Silver J (1992) Chondroitin sulfate as a regulator of neuronal patterning in the retina. Science 255:733-736.

Bruckner G, Bringmann A, Hartig W, Koppe G, Delpech B, Brauer K (1998) Acute and long-lasting changes in extracellular-matrix chondroitin-sulphate proteoglycans induced by injection of chondroitinase ABC in the adult rat brain. Exp Brain Res 121:300-310.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 23:297-308.

Caterson B, Christner JE, Baker JR, Couchman JR (1985) Production and characterization of monoclonal antibodies directed against connective tissue proteoglycans. Fed Proc 44:386-393.

Celio MR, Blumcke I (1994) Perineuronal nets--a specialized form of extracellular matrix in the adult nervous system. Brain Res Brain Res Rev 19:128-145.

Challacombe JF, Elam JS (1997) Chondroitin 4-sulfate stimulates regeneration of goldfish retinal axons. Exp Neurol 143:10-17.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403:434-439.

Clark W (1943) The problem of neuronal regeneration in the central nervous system II. The insertion of peripheral nerve stumps into the brain. 77:251-259.

Clement AM, Sugahara K, Faissner A (1999) Chondroitin sulfate E promotes neurite outgrowth of rat embryonic day 18 hippocampal neurons. Neurosci Lett 269:125-128.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214:931-933.

Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K (2002) Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J Biol Chem 277:43707-43716.

Dergham P, et al. (2002) Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570-6577.

Domeniconi M, Cao Z, Spencer T, Sivasankaran R, Wang K, Nikulina E, Kimura N, Cai H, Deng K, Gao Y, He Z, Filbin M (2002) Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. Neuron 35:283-290.

Dou CL, Levine JM (1994) Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. J Neurosci 14:7616-7628.

Dusart I, Airaksinen MS, Sotelo C (1997) Purkinje cell survival and axonal regeneration are age dependent: an in vitro study. J Neurosci 17:3710-3726.

Eng LF, Reier PJ, Houle JD (1987) Astrocyte activation and fibrous gliosis: glial fibrillary acidic protein immunostaining of astrocytes following intraspinal cord grafting of fetal CNS tissue. Prog Brain Res 71:439-455.

Evers MR, Xia G, Kang HG, Schachner M, Baenziger JU (2001) Molecular cloning and characterization of a dermatan-specific N-acetylgalactosamine 4-O-sulfotransferase. J Biol Chem 276:36344-36353.

Faissner A, Clement A, Lochter A, Streit A, Mandl C, Schachner M (1994) Isolation of a neural chondroitin sulfate proteoglycan with neurite outgrowth promoting properties. J Cell Biol 126:783-799.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24:2143-2155.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.

Fernaud-Espinosa I, Nieto-Sampedro M, Bovolenta P (1994) Differential effects of glycosaminoglycans on neurite outgrowth from hippocampal and thalamic neurones. J Cell Sci 107 (Pt 6):1437-1448.

Fouad K, Klusman I, Schwab ME (2004) Regenerating corticospinal fibers in the Marmoset (Callitrix jacchus) after spinal cord lesion and treatment with the anti-Nogo-A antibody IN-1. Eur J Neurosci 20:2479-2482.

Fournier AE, GrandPre T, Strittmatter SM (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409:341-346.

Fukuta M, Uchimura K, Nakashima K, Kato M, Kimata K, Shinomura T, Habuchi O (1995) Molecular cloning and expression of chick chondrocyte chondroitin 6-sulfotransferase. J Biol Chem 270:18575-18580.

Gilbert RJ, McKeon RJ, Darr A, Calabro A, Hascall VC, Bellamkonda RV (2005) CS-4,6 is differentially upregulated in glial scar and is a potent inhibitor of neurite extension. Mol Cell Neurosci 29:545-558.

Gotting C, Kuhn J, Zahn R, Brinkmann T, Kleesiek K (2000) Molecular cloning and expression of human UDP-d-Xylose:proteoglycan core protein beta-d-xylosyltransferase and its first isoform XT-II. J Mol Biol 304:517-528.

GrandPre T, Li S, Strittmatter SM (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417:547-551.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.

Grimpe B, Silver J (2004) A novel DNA enzyme reduces glycosaminoglycan chains in the glial scar and allows microtransplanted dorsal root ganglia axons to regenerate beyond lesions in the spinal cord. J Neurosci 24:1393-1397.

Hamai A, Hashimoto N, Mochizuki H, Kato F, Makiguchi Y, Horie K, Suzuki S (1997) Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase preferentially acting on oligosaccharides. J Biol Chem 272:9123-9130.

Hiraoka N, Nakagawa H, Ong E, Akama TO, Fukuda MN, Fukuda M (2000) Molecular cloning and expression of two distinct human chondroitin 4-O-sulfotransferases that belong to the HNK-1 sulfotransferase gene family. J Biol Chem 275:20188-20196.

Hoogewerf AJ, Cisar LA, Evans DC, Bensadoun A (1991) Effect of chlorate on the sulfation of lipoprotein lipase and heparan sulfate proteoglycans. Sulfation of heparan sulfate proteoglycans affects lipoprotein lipase degradation. J Biol Chem 266:16564-16571.

Hwang HY, Olson SK, Esko JD, Horvitz HR (2003) Caenorhabditis elegans early embryogenesis and vulval morphogenesis require chondroitin biosynthesis. Nature 423:439-443.

Ito Y, Habuchi O (2000) Purification and characterization of N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase from the squid cartilage. J Biol Chem 275:34728-34736.

Kang HG, Evers MR, Xia G, Baenziger JU, Schachner M (2001) Molecular cloning and expression of an N-acetylgalactosamine-4-O-sulfotransferase that transfers sulfate to terminal and non-terminal beta 1,4-linked N-acetylgalactosamine. J Biol Chem 276:10861-10869.

Khan A, Benboubetra M, Sayyed PZ, Ng KW, Fox S, Beck G, Benter IF, Akhtar S (2004) Sustained polymeric delivery of gene silencing antisense ODNs, siRNA, DNAzymes and ribozymes: in vitro and in vivo studies. J Drug Target 12:393-404.

Kim JE, Liu BP, Park JH, Strittmatter SM (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44:439-451.

King VR, Phillips JB, Brown RA, Priestley JV (2004) The effects of treatment with antibodies to transforming growth factor beta1 and beta2 following spinal cord damage in the adult rat. Neuroscience 126:173-183.

Kinnunen T, Raulo E, Nolo R, Maccarana M, Lindahl U, Rauvala H (1996) Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparan sulfate at the cell surface. J Biol Chem 271:2243-2248.

Kitagawa H, Uyama T, Sugahara K (2001) Molecular cloning and expression of a human chondroitin synthase. J Biol Chem 276:38721-38726.

Kitagawa H, Fujita M, Ito N, Sugahara K (2000) Molecular cloning and expression of a novel chondroitin 6-O-sulfotransferase. J Biol Chem 275:21075-21080.

Kitagawa H, Izumikawa T, Uyama T, Sugahara K (2003) Molecular cloning of a chondroitin polymerizing factor that cooperates with chondroitin synthase for chondroitin polymerization. J Biol Chem 278:23666-23671.

Kitagawa H, Tone Y, Tamura J, Neumann KW, Ogawa T, Oka S, Kawasaki T, Sugahara K (1998) Molecular cloning and expression of glucuronyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans. J Biol Chem 273:6615-6618.

Kobayashi M, Sugumaran G, Liu J, Shworak NW, Silbert JE, Rosenberg RD (1999) Molecular cloning and characterization of a human uronyl 2-sulfotransferase that sulfates iduronyl and glucuronyl residues in dermatan/chondroitin sulfate. J Biol Chem 274:10474-10480.

Kottis V, Thibault P, Mikol D, Xiao ZC, Zhang R, Dergham P, Braun PE (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. J Neurochem 82:1566-1569.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312-318.

Laabs TL, Wang H, Katagiri Y, McCann T, Fawcett JW, Geller HM (2007) Inhibiting glycosaminoglycan chain polymerization decreases the inhibitory activity of astrocyte-derived chondroitin sulfate proteoglycans. J Neurosci 27:14494-14501.

Lemons ML, Sandy JD, Anderson DK, Howland DR (2003) Intact aggrecan and chondroitin sulfate-depleted aggrecan core glycoprotein inhibit axon growth in the adult rat spinal cord. Exp Neurol 184:981-990.

Lingor P, Bahr M (2007) Targeting neurological disease with RNAi. Mol Biosyst 3:773-780.

Liu CN, Chambers WW (1958) Intraspinal sprouting of dorsal root axons; development of new collaterals and preterminals following partial denervation of the spinal cord in the cat. AMA Arch Neurol Psychiatry 79:46-61.

Lovett-Racke AE, Cravens PD, Gocke AR, Racke MK, Stuve O (2005) Therapeutic potential of small interfering RNA for central nervous system diseases. Arch Neurol 62:1810-1813.

McKeon RJ, Hoke A, Silver J (1995) Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136:32-43.

McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci 11:3398-3411.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.

McNamara JO, 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24:1005-1015.

Meiners S, Powell EM, Geller HM (1995) A distinct subset of tenascin/CS-6-PG-rich astrocytes restricts neuronal growth in vitro. J Neurosci 15:8096-8108.

Mikami T, Mizumoto S, Kago N, Kitagawa H, Sugahara K (2003) Specificities of three distinct human chondroitin/dermatan N-acetylgalactosamine 4-O-sulfotransferases demonstrated using partially desulfated dermatan sulfate as an acceptor: implication of differential roles in dermatan sulfate biosynthesis. J Biol Chem 278:36115-36127.

Misgeld T, Kerschensteiner M (2006) In vivo imaging of the diseased nervous system. Nat Rev Neurosci 7:449-463.

Misgeld T, Nikic I, Kerschensteiner M (2007) In vivo imaging of single axons in the mouse spinal cord. Nat Protoc 2:263-268.

Moon LD, Asher RA, Rhodes KE, Fawcett JW (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. Nat Neurosci 4:465-466.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew MV (2006) Essential protective roles of reactive astrocytes in traumatic brain injury. Brain 129:2761-2772.

Nadanaka S, Clement A, Masayama K, Faissner A, Sugahara K (1998) Characteristic hexasaccharide sequences in octasaccharides derived from shark cartilage chondroitin sulfate D with a neurite outgrowth promoting activity. J Biol Chem 273:3296-3307.

Nishizuka M, Ikeda S, Arai Y, Maeda N, Noda M (1996) Cell surface-associated extracellular distribution of a neural proteoglycan, 6B4 proteoglycan/phosphacan, in the olfactory epithelium, olfactory nerve, and cells migrating along the olfactory nerve in chick embryos. Neurosci Res 24:345-355.

Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, Michalovich D, Simmons DL, Walsh FS (2000) Inhibitor of neurite outgrowth in humans. Nature 403:383-384.

Properzi F, Carulli D, Asher RA, Muir E, Camargo LM, van Kuppevelt TH, ten Dam GB, Furukawa Y, Mikami T, Sugahara K, Toida T, Geller HM, Fawcett JW (2005) Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. Eur J Neurosci 21:378-390.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. Nat Biotechnol 22:326-330.

Richardson PM, McGuinness UM, Aguayo AJ (1980) Axons from CNS neurons regenerate into PNS grafts. Nature 284:264-265.

Sango K, Oohira A, Ajiki K, Tokashiki A, Horie M, Kawano H (2003) Phosphacan and neurocan are repulsive substrata for adhesion and neurite extension of adult rat dorsal root ganglion neurons in vitro. Exp Neurol 182:1-11.

Scherr M, Morgan MA, Eder M (2003) Gene silencing mediated by small interfering RNAs in mammalian cells. Curr Med Chem 10:245-256.

Schmalfeldt M, Bandtlow CE, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR (2000) Brain derived versican V2 is a potent inhibitor of axonal growth. J Cell Sci 113 (Pt 5):807-816.

Silbert JE, Sugumaran G (2002) Biosynthesis of chondroitin/dermatan sulfate. IUBMB Life 54:177-186.

Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, Christ F, Sansig G, van der Putten H, Schwab ME (2003) Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. Neuron 38:201-211.

Smith-Thomas LC, Stevens J, Fok-Seang J, Faissner A, Rogers JH, Fawcett JW (1995) Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors. J Cell Sci 108 (Pt 3):1307-1315.

Smith GM, Strunz C (2005) Growth factor and cytokine regulation of chondroitin sulfate proteoglycans by astrocytes. Glia 52:209-218.

Snow DM, Brown EM, Letourneau PC (1996) Growth cone behavior in the presence of soluble chondroitin sulfate proteoglycan (CSPG), compared to behavior on CSPG bound to laminin or fibronectin. Int J Dev Neurosci 14:331-349.

Talts U, Kuhn U, Roos G, Rauch U (2000) Modulation of extracellular matrix adhesiveness by neurocan and identification of its molecular basis. Exp Cell Res 259:378-388.

Taylor KR, Gallo RL (2006) Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. Faseb J 20:9-22.

Tester NJ, Plaas AH, Howland DR (2007) Effect of body temperature on chondroitinase ABC's ability to cleave chondroitin sulfate glycosaminoglycans. J Neurosci Res 85:1110-1118.

Uchimura K, Kadomatsu K, Fan QW, Muramatsu H, Kurosawa N, Kaname T, Yamamura K, Fukuta M, Habuchi O, Muramatsu T (1998) Mouse chondroitin 6-sulfotransferase: molecular cloning, characterization and chromosomal mapping. Glycobiology 8:489-496.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.

Windle WF, Chambers WW (1950) Regeneration in the spinal cord of the cat and dog. J Comp Neurol 93:241-257.

www.ncbi.nlm.nih.gov/sites/entrez?db=gene (2008) Entrez Gene. In.

www.spinalcord.uab.edu (2008) Spinal Cord Injury Facts & Figures at a Glance 2008. In.

Yamada H, Fredette B, Shitara K, Hagihara K, Miura R, Ranscht B, Stallcup WB, Yamaguchi Y (1997) The brain chondroitin sulfate proteoglycan brevican associates with astrocytes ensheathing cerebellar glomeruli and inhibits neurite outgrowth from granule neurons. J Neurosci 17:7784-7795.

Yamagata T, Saito H, Habuchi O, Suzuki S (1968) Purification and properties of bacterial chondroitinases and chondrosulfatases. J Biol Chem 243:1523-1535.

Yeo TK, Brown L, Dvorak HF (1991) Alterations in proteoglycan synthesis common to healing wounds and tumors. Am J Pathol 138:1437-1450.

Zhang Y, Tohyama K, Winterbottom JK, Haque NS, Schachner M, Lieberman AR, Anderson PN (2001) Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. Mol Cell Neurosci 17:444-459.