DELIVERY OF CDC42, RAC1, AND BRAIN-DERIVED NEUROTROPHIC FACTOR TO PROMOTE AXONAL OUTGROWTH AFTER SPINAL CORD INJURY

A Dissertation
Presented to
The Academic Faculty

by

Anjana Jain

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Biomedical Engineering

Georgia Institute of Technology
August 2007

DELIVERY OF CDC42, RAC1, AND BRAIN-DERIVED NEUROTROPHIC FACTOR TO PROMOTE AXONAL OUTGROWTH AFTER SPINAL CORD INJURY

Approved by:

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

Dr. Susann M. Brady-Kalnay
Department of Molecular Biology
Case Western Reserve University

Dr. Michelle C. LaPlaca
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Robert J. McKeon
Department of Cell Biology
Emory University

Dr. Todd C. McDevitt
School of Biomedical Engineering
Georgia Institute of Technology

Date Approved: August 06, 2007

"***samyag-darśana-jnāna-cāritrāni-moksamārgah***"

-from *Tattvārtha Sūtra*

Translation- "The enlightened world-view, enlightened knowledge and enlightened conduct are the path to liberation"

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my advisor, Dr. Ravi Bellamkonda, for his guidance and encouragement in the accomplishment of this work. He provided an environment that allowed me to develop into a researcher and a contributor to the scientific community. For this opportunity, I will always be grateful.

I would also like to acknowledge my committee members, Dr. Michelle LaPlaca, Dr. Todd McDevitt, Dr. Susann Brady-Kalnay and Dr. Robert McKeon, for their "open-door policy", providing me with invaluable insight and advice, protein, and facilities that allowed this study to reach its fruition.

For several years, 24 hours a day, 7 days a week, your life is spent as a graduate student in a laboratory, which can be a daunting task; however, I was fortunate to be surrounded by supportive labmates, who created an environment where I could spend endless hours working. To the members of the Biomaterials, Cellular and Tissue Engineering Laboratory (BCTE), I would like to express my appreciation. As one of the younger members of the lab, you mentored me to become a better scientist, a critical thinker, as well as befriended me. To the Neurological Biomaterials and Therapeutics Laboratory (NeuroBAT), both past and present, we shared many a laughs during our intellectual conversations. I will have fond memories of the times we have shared both in and out of the laboratory. I sincerely wish you the best of luck in your future endeavors in the laboratory and beyond. I also wish to express my appreciation to the administrative staff, who always provided a helpful hand and a friendly smile.

Lastly, words cannot express the unconditional emotional and financial support my parents and my big di, Rakhi, have provided me. Through my journey, I have found you by my side; providing me your unlimited energy and strength when there was a time I could not find my own.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS i

LIST OF FIGURES xi

SUMMARY xiii

CHAPTER

1 INTRODUCTION 1

1.1 STATEMENT OF PROBLEM 1

1.2 HYPOTHESIS 2

1.3 OBJECTIVES 4

1.4 REFERENCES 5

2 THERAPEUTIC STRATEGIES AFTER SPINAL CORD INJURY 6

2.1 EXTRINSIC STRATEGIES FOR AXONAL REGENERATION 8

2.1.1 Myelin-associated Inhibitors 8

2.1.2 Chondroitin Sulfate Proteoglycans 10

2.2 INTRINSIC STRATEGIES TO ALLEVIATE INHIBITORY ENVIRONMENT AT THE SITE OF INJURY 11

2.2.1 Rho GTPases 11

2.2.2 Cyclic AMP 12

2.2.3 Neurotrophic Factors Stimulate Process Extension 13

2.3 CELL TRANSPLANTATION FOR CELL-SCAFFOLD CONSTRUCTS: COMBINING SUBSTRATES WITH STIMULI FOR REGENERATION 14

2.3.1 Schwann Cells 14

2.3.2 Olfactory Ensheathing Glia 15

2.3.3 Macrophages 16

2.3.4 Stem Cells and Neural/Glia Progenitor Cells 17

2.3.5 Astrocytes 18

2.4 GROWTH PERMISSIVE SUBSTRATES TO ACTIVELY SUPPORT GROWING AXONS 19

2.4.1 Spatial Control: Permissive Bioactive Hydrogel Scaffolds for Enhanced Regeneration 20

2.4.2 Spatial Control: Contact Guidance (Fibers) as a Strategy to Promote Regeneration 24

2.5 TEMPORALLY CONTROLLING THE RELEASE OF PROTEINS 27

2.5.1 Temporal Control: Osmotic Pumps Release Protein to Encourage Axonal Outgrowth 28

2.5.2 Temporal Control: Slow Release of Trophic Factors Using Microspheres 30

2.5.3 Temporal Control: Lipid Microtubule for Sustained Release of Stimulatory Trophic Factors 31

2.5.4 Temporal Control: Demand Driven Release of Trophic Factors 32

2.6 TRANSDUCTION OF PROTEINS OR TRANSGENES 33

2.6.1 Peptide, Non-viral Delivery Systems 33

2.6.2 Viral-based Delivery Systems 34

2.7 SPINAL CORD INJURY MODELS 37

2.7.1 Contusion Injury 37

2.7.2 Complete Transection Injury 38

2.7.3 Lateral Hemisection Injury 38

2.7.4 Doral Over-hemisection Injury 38

2.8 CONCLUSIONS 39

2.9 REFERENCES 40

3 MODULATION OF RHO GTPASES TO OVERCOME CSPG-DEPENDENT INHIBITION ON NEURITE EXTENSION *IN VITRO* 50

3.1 INTRODUCTION 51

3.2 MATERIALS AND METHODS 53

3.2.1 Surface Modification of Tissue Culture Dishes 53

3.2.2 NG108-15 Cell Neurite Outgrowth Assays 54

3.2.3 DRG Neurite Outgrowth Assays 56

3.2.4 Quantification of Neurite Crossing 56

3.2.5 Statistical Analysis 57

3.3 RESULTS 57

3.3.1 Percent Ratio of Neurites Crossing After Transduction of Rho GTPases 57

3.3.2 Optimal Dosage for Percent Ratio of Neurite Crossing 60

3.3.3 Effect of Chariot® Transduction System 61

3.3.4 Transduction of Rho GTPases into DRGs 63

3.4 DISCUSSION 66

3.5 ACKNOWLEDGEMENTS 71

3.6 REFERENCES 71

4 DELIVERY AND CHARACTERIZATION OF BDNF, CA-CDC42, AND CA-RAC1 TO PROMOTE AXONAL OUTGROWTH THROUGH CSPG-INHIBITORY REGIONS AFTER SPINAL CORD INJURY 75

4.1 INTRODUCTION 76

4.2 MATERIALS AND METHODS 80

4.2.1 Agarose Gelling Temperature 80

4.2.2 Neurite Extension of DRGs in 3D Agarose Gels 81

4.2.3 Fabrication and Loading of Lipid Microtubules for Sustained Release of CA-Cdc42, CA-Rac1, and BDNF 81

4.2.4 Characterization of Microtubule Mediated Sustained Release of BDNF *In Vitro* 82

4.2.5 Conjugation of Rhodamine to BDNF 83

4.2.6 Cooling System for *In Situ* Gelling Agarose 83

4.2.7 Implantation of Agarose-Protein Scaffolds in a Dorsal Over-hemisection Model *In Vivo* 84

4.2.8 Anterograde Neuronal Tracer Injection into the Corticospinal Tract 85

4.2.9 Immunohistochemistry and Histological Evaluation of Explanted Spinal Cords and Hydrogels 86

4.2.10 Quantitative Analysis of Cellular and Molecular Response to Scaffold and Protein 88

4.2.11 Quantification of Average Lesion Area 89

4.2.12 Axonal Outgrowth Through Inhibitory Regions Proximal to Lesion Site 90

4.2.13 Quantification of BDA^{+} Axons 91

4.2.14 Behavioral Analysis 92

4.2.15 Statistical Analysis 92

4.3 RESULTS 93

4.3.1 Characterization of Agarose Hydrogels 93

4.3.2 Characterization of BDNF Release 96

4.3.3 *In Situ* Gelling Hydrogel in Spinal Cord Cavity 98

4.3.4 Cellular and Molecular Inflammatory Response 99

4.3.5 Crossing of Axons Through Inhibitory Regions 105

4.3.6 Sprouting Axons After Implantation of Hydrogel/Protein Microtubule Delivery System 108

4.3.7 Behavioral Analysis 113

4.4 DISCUSSION 118

4.5 ACKNOWLEDGEMENTS 127

4.6 REFERENCES 127

5 CONCLUSIONS 131

6 FUTURE PERSPECTIVES 139

6.1 OPTIMIZATION OF DOSAGE 139

6.2 OPTIMIZATION OF DELIVERY VEHICLE 140

6.3 MODIFICATION OF SCAFFOLD 140

6.4 DURATION OF *IN VIVO* STUDY 141

6.5 SHORT-TERM STUDY OF INFLAMMATORY RESPONSE 141

6.6 MULTI-FACTORIAL APPROACH 141

REFERENCES 143

LIST OF FIGURES

Page

Figure 3.1: Percent ratio of neurites crossing for controls, Rho GTPase mutant proteins and C3 transferase 58

Figure 3.2: NG108-15 cells transduced with mutant Rho GTPases and C3 59

Figure 3.3: CA-Cdc42 dose response curve for NG108-15 cells 60

Figure 3.4: Percent ratio of neurites crossing after CA-Rac1 treatment with and without Chariot® 61

Figure 3.5: C3 transferase with and without Chariot® 62

Figure 3.6: DRG neurite preference for a substrate 64

Figure 3.7: DRGs transduced with mutant Rho GTPases and C3 65

Figure 4.1: Gel cooling system 84

Figure 4.2: Method for quantitative analysis of immunostained spinal cord sections 89

Figure 4.3: Schematic of axon quantification in CS-56$^+$-inhibitory regions 90

Figure 4.4: Schematic of quantification of percent of number of BDA$^+$ axons from beginning of lesion site to 4 mm proximal 92

Figure 4.5: Gelling temperatures for various agarose gels and concentrations 94

Figure 4.6: Micrograph images of DRGs cultured in different agarose gels after 48 hrs at 5x magnification 95

Figure 4.7: Diffusion of BDNF 97

Figure 4.8: Images demonstrating agarose injected into spinal cord cavity 98

Figure 4.9: GFAP and CS-56 expression at the lesion site 100

Figure 4.10: GFAP fluorescent intensity from the interface into the spinal cord 101

Figure 4.11: CS-56 fluorescent intensity from the interface into the spinal cord 102

Figure 4.12: Comparison of the number of ED-1$^+$ macrophages/reactive microglia around the lesion area 103

Figure 4.13: Average lesion area of the controls and treated spinal cords 104

Figure 4.14: BDA^+ axons and $CS\text{-}56^+$ inhibitory regions 106

Figure 4.15: Percentage of axons in $CS\text{-}56^+$ inhibitory regions 107

Figure 4.16: BDA^+ axons in CST 109

Figure 4.17: Axonal retraction from the lesion site in the treated and control spinal cords 110

Figure 4.18: Percent of axonal outgrowth towards the lesion site 112

Figure 4.19: $NF\text{-}160^+$ fluorescent images 114

Figure 4.20: Quantitative analysis of NF-160 intensity for stained spinal cords 115

Figure 4.21: Grid walk test at 3 and 5 weeks post-injury 116

Figure 4.22: Beam walk test at 3 and 5 weeks post-injury 117

SUMMARY

Injury severs the axons in the spinal cord causing permanent functional loss. After injury, a series of events occur around the lesion site, including the deposition of growth cone inhibitory astroglial scar tissue containing chondroitin sulfate proteoglycan (CSPG)- rich regions. It is important to encourage axons to extend through these inhibitory regions for regeneration to occur. The work presented in this dissertation investigates the effect of three proteins, constitutively active (CA)-Cdc42, CA-Rac1, and brain-derived neurotrophic factor (BDNF) on axonal outgrowth through CSPGs-rich inhibitory regions after spinal cord injury (SCI). Cdc42 and Rac1 are members of the Rho GTPase family and BDNF is a member of the neurotrophin sub-family. These three proteins affect the actin cytoskeleton dynamics. Therefore, Cdc42, Rac1, and BDNF promote axonal outgrowth.

The effect of CA-Cdc42 and CA-Rac1 on neurite extension through CSPG regions was determined in an *in vitro* model. Rac1 and Cdc42's ability to modulate CSPG-dependent inhibition has yet to be explored. In this study, a stripe assay was utilized to examine the effects of modulating all three Rho GTPases on neurite extension across inhibitory CSPG lanes. Alternating laminin (LN) and CSPG lanes were created and NG108-15 cells and E9 chick dorsal root ganglions (DRGs), were cultured on the lanes. Using the protein delivery agent Chariot®, the neuronal response to exposure of CA and dominant negative (DN) Rho GTPases, along with the bacterial toxin C3, was determined by quantifying the percent ratio of neurites crossing the CSPG lanes. CA-Cdc42, CA-Rac1, and C3 transferase significantly increased the number of neurites

crossing into the CSPG lanes compared to the negative controls for both the NG108-15 cells and the E9 chick DRGs. We also show that these mutant proteins require the delivery vehicle, Chariot®, to enter the neurons and affect neurite extension. Therefore, activation of Cdc42 and Rac helps overcome the CSPG-dependent inhibition of neurite extension.

In an *in vivo* study, CA-Cdc42 and CA-Rac1 were locally delivered into a spinal cord cavity. Additionally, BDNF was delivered to the lesion site, either individually or in combination with either CA-Cdc42 or CA-Rac1. The dorsal over-hemisection model was utilized, creating a ~2mm defect that was filled with an *in situ* gelling hydrogel scaffold containing lipid microtubules loaded with the protein(s) to encourage axons. The lipid microtubules enable slow release of proteins while the hydrogel serves to localize them to the lesion site and permit axonal growth. The results from this study demonstrate that groups treated with BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 had significantly higher percentage of axons from the corticospinal tract (CST) that traversed the CSPG-inhibitory regions, as well as penetrate the glial scar compared to the untreated and agarose controls. Although axons from the CST tract did not infiltrate the scaffold-filled lesion, NF-160^{+} axons were observed in the scaffold. Treatment with BDNF, CA-Cdc42, and CA-Rac1 also reduced the inflammatory response, quantified by analyzing GFAP and CS-56 intensity for reactive astrocytes and CSPGs, respectively, at the interface of the scaffold and host tissue. Therefore, the local delivery of CA-Cdc42, CA-Rac1 and BDNF, individual and combination demonstrated the ability of axons to extend through CSPG inhibitory regions, as well as reduce the glial scar components.

CHAPTER 1

INTRODUCTION

1.1 STATEMENT OF PROBLEM

There is currently no successful therapeutic strategy for axonal regrowth through the inhibitory regions, specifically chondroitin sulfate proteoglycan-rich regions, after spinal cord injury.

Injury to the central nervous system (CNS), especially to the spinal cord severely affects the quality of life. Eleven thousand people annually suffer from spinal cord injury (SCI) in the United States alone (sci-info-pages.com March 2002). There are currently 250,000 people in the United States that are adapting to life after SCI. The effect of SCI on patients can vary depending upon the location and severity of the injury. Individuals may experience loss of sensation, muscle paralysis, sexual dysfunction, and loss of bladder and bowel control. Along with the physical and emotional hardships after SCI, the cost of receiving treatment is a burden on the patient, as well as their family, with medical costs that can approach $1,000,000 per patient in the first year alone.

There is a great need to find a tissue engineered therapeutic solution to regenerate axons in the CNS. The current options for therapeutic clinical treatment in the CNS after injury are limited. Anti-inflammatory agents, such as methylprednisolone, are administered after injury, but these do not provide any regenerative stimuli. The main goals of the anti-inflammatory agents are to minimize pain and prevent further damage to

the spinal cord. Surgical intervention is currently a subject of intense debate, with differing opinions on when surgery should be performed after injury. Although surgery may be performed to stabilize the spine by using a rigid brace in order to prevent further damage it does not typically provide any regenerative benefits.

There are ongoing clinical trials that involve cellular transplantation of macrophages or stem cells, and testing the use of trophic factors. Studies have investigated implantation of scaffolds involving cell transplantation, hydrogel scaffolds, and fibers, as well as various delivery vehicles, such as osmotic pumps, microspheres, and lipid microtubules. These studies in animal models have shown functional improvement; however, high percentage of axons that infiltrate the spinal cord distal to the lesion site needs to be enhanced. This study investigates two proteins, Cdc42 and Rac1, in combination of a neurotrophic factor, BDNF, to potentially improve axonal outgrowth through the inhibitory regions.

1.2. HYPOTHESIS

Complete functional recovery after physical injury in the CNS remains a challenge. Overcoming the challenge to promote axonal outgrowth requires: 1) negating the effects of the glial scar that forms after injury, which contains inhibitory cues that prevent the damaged axons from reforming lost connections; 2) encouraging axonal outgrowth despite the presence of the glial scar; and 3) delivering therapeutic agents without causing further damage to the spinal cord. We believe spatio-temporal control of the therapeutic agents is a critical component to achieve axonal outgrowth through the inhibitory regions in the spinal cord.

The failure of axonal outgrowth through inhibitory regions after injury converge on the Rho GTPases, Cdc42, Rac, and Rho, which are responsible for filopodial, lamellopodial, and growth cone collapse, respectively (Nobes and Hall 1995). Studies have shown that when Nogo receptor and its coreceptor, $p75^{NTR}$, bind to the inhibitory molecule ligands, the signaling pathway for Rho is activated (Niederost et al. 2002; Schweigreiter et al. 2004). Cross-talk occurs between the three proteins (Kjoller and Hall 1999; Yuan et al. 2003). Specifically, the activation of Rho causes a decrease in the levels of Cdc42 and Rac, thus leading to growth cone collapse. Most of the research has focused on inhibiting Rho or the downstream effectors. We believe that the activation of Cdc42 and Rac1 should promote filopodial and lamellopodial extension, thereby encouraging axonal outgrowth through the inhibitory regions.

It is important to achieve spatial and temporal control of the delivered proteins at the injury site. For spatial control, an *in situ* gelling agarose hydrogel scaffold will: 1) allow conformal filling of the defect created after injury, 2) provide the site for local deliver of the proteins, and 3) allow for axonal infiltration. Most studies use the osmotic pump to deliver trophic factors and proteins to the lesion site; however, a large amount of the protein is removed by bodily fluids. We believe that the use of a slow release delivery vehicle, the lipid microtubules, contained within the scaffold will allow the proteins to diffuse into the surrounding spinal cord tissue and be accessible to the neurons and axons over the desired duration.

1.3. OBJECTIVES

The overall goal of the work described in this thesis is to deliver proteins, activated Cdc42, activated Rac1, and BDNF to encourage axonal outgrowth through the inhibitory regions, particularly regions rich in CSPGs.

To determine whether Cdc42, Rac1, and BDNF promote axonal outgrowth, the following objectives were set:

1. Development of a model that shows *in vitro* that the transduction of constitutively active forms of Cdc42 and Rac1 can encourage neurites to extend into CSPG-inhibitory regions.
2. Development and characterization of a delivery system for Cdc42, Rac1, and BDNF, which are transduced to promote axonal outgrowth through inhibitory regions *in vivo*. This will be achieved by:
    a. Development of a hydrogel scaffold that conformally fills the spinal cord defect and supports axonal infiltration and outgrowth.
    b. Development and characterization of local, slow delivery of the proteins using the lipid microtubule delivery system embedded in the hydrogel scaffold.

1.4. REFERENCES

Kjoller L, Hall A. 1999. Signaling to Rho GTPases. Exp Cell Res 253(1):166-179.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE. 2002. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22(23):10368-10376.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Schweigreiter R, Walmsley AR, Niederost B, Zimmermann DR, Oertle T, Casademunt E, Frentzel S, Dechant G, Mir A, Bandtlow CE. 2004. Versican V2 and the central inhibitory domain of Nogo-A inhibit neurite growth via p75NTR/NgR-independent pathways that converge at RhoA. Mol Cell Neurosci 27(2):163-174.

sci-info-pages.com. March 2002. Spinal Cord Injury Facts & Statistics. http://wwwsci-info-pagescom/factshtml. May 2007.

Yuan XB, Jin M, Xu X, Song YQ, Wu CP, Poo MM, Duan S. 2003. Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat Cell Biol 5(1):38-45.

CHAPTER 2

THERAPEUTIC STRATEGIES AFTER SPINAL CORD INJURY

Physical injury to the spinal cord can lead to permanent functional loss due to the lack of spontaneous regeneration capabilities in the CNS. The relative inability to regenerate is possibly due to the body's wound healing response. After injury to the CNS, a glial scar forms and there is a migration of macrophages/reactive microglia to the wound site to remove the debris produced (Fawcett and Asher 1999). While these cells are present at the injury site, they secrete proteins, such as cytokines, that activate other local cells. Astrocytes are one of these cells that become reactive after the release of cytokines. At the injury site, there are inhibitory molecules that are up-regulated or exposed making the environment non-permissive for nerve regeneration. Astrocytes, for example, up-regulate chondroitin sulfate proteoglycans (CSPGs) and oligodendrocyte debris is exposed, as well as myelin-related proteins, such as myelin associated glycoprotein (MAG) (McKerracher et al. 1994; Mukhopadhyay et al. 1994), NOGO (Niederost et al. 2002), and oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al. 2002). Other inhibitory molecules are also present in the glial scar; however, the non-regenerative environment has been mainly attributed to CSPGs, MAG, NOGO, and OMgp.

The current clinical challenge is to obtain regeneration with accompanying functional recovery after injury in the spinal cord. The crux of the approaches currently under development to promote regeneration in the CNS involves manipulating events in a

controlled manner. Research to promote axonal outgrowth ranges from delivery of neurotrophic factors to proteins involved in actin cytoskeleton dynamics or alters the microenvironment. To deliver these axonal promoting agents to the spinal cord, cellular transplantation, the utilization of scaffolds, and various temporal delivery vehicles, such as osmotic pumps and microparticles have been implemented.

The research currently investigated to promote axonal regrowth can be divided into two main strategies: extrinsic and intrinsic. The extrinsic strategy alters the microenvironment at and around the lesion site to promote axonal sprouting. An example of this is to deliver siRNA that knocks-down inhibitory molecules, such as neurocan, a CSPG that is up-regulated after injury. The second strategy involves intrinsically modifying cells to overcome the inhibitory nature of the glial scar. An example of utilizing an intrinsic strategy is to modulate the Rho GTPases, which affect the actin cytoskeleton dynamics. Three different areas are being research within the two strategies to promote axonal outgrowth. The first area is to provide permissive bioactive substrates for axonal outgrowth. For example, nerves are anchorage-dependent and the design of a substrate or bridge between the severed ends is an opportunity to present the correct spatial and temporal cues to both guide and stimulate axonal growth across the nerve gap. The second area is to deliver trophic factors in order to promote and stimulate axonal growth involving the application of specific factors known as neurotrophic factors in a spatially and temporally controlled manner *in vivo*. The third area is to alleviate signaling due to the inhibitory entities present in the extracellular environment to allow axons to regenerate between the proximal and distal ends. Any inhibitory cellular responses and/or any inhibitory cues that may be generated after injury must be

modulated such that they do not interfere in any putative regenerative attempt after injury. This third aspect is especially critical in the CNS where an extremely inhibitory glial scar is generated at the distal nerve segment, often leading to regenerative failure in the CNS. There are a series of technologies and challenges that are encountered in achieving this goal and they are discussed below.

2.1. EXTRINSIC STRATEGIES FOR AXONAL REGENERATION

2.1.1. Myelin-associated Inhibitors

As mentioned previously, Nogo, MAG, and OMgp are bound to the surface of myelin and are partially responsible for regenerative failure. Studies have been performed to determine whether regenerative and functional recovery can be regained by knocking-out these myelin-associated proteins. Nogo has been the most extensively studied. This protein is a member of the Reticulon Family and has two transmembrane domains. There are three isoforms: Nogo-A, -B, and –C, which are similar in sequence at the C-terminus (Schweigreiter and Bandtlow 2006). This inhibitory molecule binds to the Nogo receptor (NgR). Another receptor, $p75^{NTR}$, which is a neurotrophin receptor, behaves as a co-receptor to NgR, thus activating a signaling-transduction pathway as a receptor complex (Wang et al. 2002a; Wong et al. 2002). Studies have been performed examining the contribution of Nogo and its receptors, NgR and $p75^{NTR}$, in the lack of axonal regeneration after injury. Contradictory results have been reported regarding the inhibition of Nogo and its affect on axonal regeneration. Genetically engineered mice that were Nogo-deficient demonstrated that in the absence of this inhibitory molecule, significant axonal regeneration did not occur after SCI (Zheng et al. 2003). In another

study, NgR and $p75^{NTR}$ were individually knocked down in mice and failed to exhibit enhanced axonal regeneration (Zheng et al. 2005), demonstrating that another inhibitory molecule and not Nogo may be the major contributor in inhibiting axonal outgrowth through injured spinal cord. However, there have been studies published by the Strittmatter Laboratory that have shown that a peptide antagonist, developed from Nogo sequences, which competitively binds to NgR promoted axonal regeneration and improved functional recovery (GrandPre et al. 2002; Li and Strittmatter 2003).

The second myelin-associated protein, MAG, a member of the immunoglobulin gene superfamily, is a transmembrane protein. MAG was the first of the three myelin-associated inhibitors identified as an inhibitor to axonal regeneration (McKerracher et al. 1994; Mukhopadhyay et al. 1994). However, the role of MAG as a major contributor to inhibition has been questioned. MAG is also present in the peripheral nervous system where axonal regeneration does not appear to be hindered by inhibitory molecules as in the CNS(Aguayo et al. 1981). This begs the question as to its role in inhibition. Although studies have shown that MAG is inhibitory for outgrowth *in vitro*, a study demonstrated that in MAG knocked-out mice, axonal outgrowth was similar to wild-type animals, which was not significant (Bartsch et al. 1995).

OMgp was the most recently identified myelin-associated inhibitor (Kottis et al. 2002). This protein is glycosylphosphatidylinositol anchored and has leucine repeats (Vourc'h et al. 2003). *In vitro* studies have shown that OMgp is inhibitory towards neurite outgrowth(Wang et al. 2002b); however, extensive research on its influence on axonal regeneration *in vivo* has not yet been conducted.

2.1.2. Chondroitin Sulfate Proteoglycans

The contradictory evidence of axonal regenerative capabilities after SCI in myelin-associated protein deficient mice has lead to the belief that CSPGs are the major inhibitors present in the microenvironment preventing axons from crossing the lesion site into the caudal segments of the spinal cord. After injury, astrocytes and oligodendrocyte precursor cells secrete CSPGs (Morgenstern et al. 2002), as well as macrophages and reactive microglia (Jones et al. 2002) into the glial scar matrix. CSPGs consist of a protein core and glycosaminoglycans (GAGs) side chains. It has been demonstrated that both components contribute to the inhibitory nature of the macromolecule (Hoke and Silver 1996; Morgenstern et al. 2002).

Currently, many studies are investigating the removal of the inhibitory nature of the CSPGs through enzymatic degradation. In particular, chondroitinase ABC (chABC) is a bacterial enzyme that cleaves the GAG side chains into disaccharide units. Consequently, studies have shown that after treating the CSPGs with chABC, axonal regeneration could occur and extend through the inhibitory glial scar region into the distal nerve end (Bradbury et al. 2002; McKeon et al. 1995; Zuo et al. 1998; Zuo et al. 2002). Although this bacterial enzyme has been introduced into rodent models to regenerate axons after SCI, it is a concern that chABC contains impurities and elicit an immune response. Recently, a study was published where reactive astrocytes were genetically modified to express chABC. Compared to the wild type, the axons were able to penetrate through the proximal region of the glial scar and enter into the lesion site (Cafferty et al. 2007). Although administration of chABC after SCI has shown potential as a possible therapeutic intervention, one must wonder about the effect of chABC on CSPGs

deposited during developmental stages to maintain the tracts and are located in the perineuronal nets. Other strategies that are currently being investigated involve the use of ODNs and siRNAs to prevent the production of CSPGs by reactive astrocytes after injury. If this is successful, the CSPGs deposited for tract maintenance will be preserved and only the negative CSPGs will be knocked-out.

2.2. INTRINSIC STRATEGIES TO ALLEVIATE INHIBITORY ENVIRONMENT AT THE SITE OF INJURY

2.2.1. Rho GTPases

Neurites extend from the neuronal body and have a growth cone at the tip. The role of the growth cone is to read the environmental cues and decide which direction the neurite will grow towards. The growth cone extends filopodia and lamellipodia to read the cues. Rho GTPases are involved in actin cytoskeleton dynamics, specifically in promoting filopodial and lamellipodial extension (Nobes and Hall 1995). There are three main members of the Rho GTPase family, Cdc42, Rac, and Rho. Cdc42 and Rac induce filopodial and lamellipodial outgrowth, respectively, and activation of Rho results in growth cone collapse. In the glial scar the inhibitory molecules prevent the extension of the filopodia and lamellipodia and induce growth cone collapse. Studies have shown that MAG and Nogo activate the signaling pathway that converges on Rho, thus causing growth cone collapse. Therefore, it is necessary to mask or remove these negative components from the microenvironment in order to support axonal outgrowth. Mutant Rho GTPases derivatives have been constructed (Coso et al. 1995; Kozma et al. 1995). When the Rho GTPases are GTP-bound they are in the active state and they are inactive

when bound to GDP. The two derivates are constitutively active (CA) and dominant negative (DN). Proteins that are constitutively active are always GTP-bound, therefore, in the active state. Dominant negative Rho GTPases are in the inactive conformation. Studies have utilized these derivatives to investigate how neurite extension is affected when in contact with inhibitory molecules. Protein transduction of these mutant derivatives is one of the methods utilized to promote and stimulate axonal regeneration. By intracellularly modulating levels of activated GTPase, the growth cone can be manipulated to extend and grow into an inhibitory environment. Modulating Rho GTPases is one of the ways to overcome glial scar inhibition through protein transduction (Dubreuil et al. 2003; Jain et al. 2004; Monnier et al. 2003; Winton et al. 2002). By modulating Rho GTPases levels in the neurons and elevating the concentration, the inhibitory effects of the glial scar will be masked and the growth cone will lead the axon towards the distal nerve ending.

2.2.2. Cyclic AMP

Other molecules that have been used to encourage neurite outgrowth in the face of inhibitory signals are cAMP and calcium in the CNS (Mattson et al. 1988). It has been shown that the modulation of cAMP, through the use of active and inactive analogs, can encourage neurite outgrowth through inhibitory substrates (Bandtlow 2003). The activation of the signal transduction pathway by cAMP shows that axonal regeneration can be stimulated and promoted (Qiu et al. 2002). *In vivo* studies have also shown that increasing cAMP levels will promote axonal outgrowth (David and Lacroix 2003). There

has been speculation that cAMP is proximal to the Rho GTPases on the same signaling transduction pathway, thus suggesting that cAMP can affect actin cytoskeleton dynamics.

2.2.3. Neurotrophic Factors Stimulate Process Extension

Neurotrophic factors have an important role in neural development and in adult life for axonal regeneration. Neurotrophins, nerve growth factor (NGF), BDNF, and neurotrophin-3 (NT-3), are a specific family of neurotrophic factors, which promote regeneration in the nervous system. NGF was the first neurotrophin discovered in a study that investigated the effects of lost target tissues on sensory and motor neuron survival (Levi-Montalcini 1987). BDNF was the next to be discovered followed by NT-3. The neurotrophins bind to two classes of receptors, the tropomyosin receptor kinase (Trk) and the p75 neurotrophin receptor ($p75^{NTR}$). The Trk receptors are members of the receptor tyrosine kinase (RTK) family and there are three subtypes, TrkA, TrkB, and TrkC. NGF binds to TrkA and activates its signaling pathway, while TrkB and TrkC bind to BDNF, and NT-3, respectively (Hennigan et al. 2007). It has been suggested that when the trophic factors bind to Trk receptors, the signaling pathways for neuronal survival are activated, where as activation of $p75^{NTR}$ induces cell death (Hennigan et al. 2007). This is correlated with the studies that demonstrate $p75^{NTR}$ role in the activation of Rho. In the CNS, BDNF and NT-3 have been investigated for their regenerative capabilities. Several studies, which will be discussed later, have shown that after administration of BDNF or NT-3, axonal regeneration was promoted in spinal cord injured adult animals.

2.3. CELL TRANSPLANTATION FOR CELL-SCAFFOLD CONSTRUCTS: COMBINING PERMISSIVE SUBSTRATES WITH STIMULI FOR REGENERATION

The birth of cell transplantation began with the discovery in the early 1900s by Ramon y Cajal; axons could infiltrate the peripheral nerve transplanted in the CNS. Cell transplantation techniques are an elegant way to combine two promising strategies to elicit regeneration: permissive substrates and spatio-temporally controlled delivery of trophic factors at the site of injury.

Olfactory ensheathing glia (OEG), fetal tissue, stem cells/neuronal precursor cells, Schwann cells, and macrophages are cells that have been transplanted in the spinal cord after injury, alone and in conjunction with each other and peripheral nerve grafts. These cells provide both trophic cues, as well as physical contact guidance type cues in promoting regeneration as described below. The use of cells, such as glia, utilizes the strategy that modulates intrinsic mechanisms to promote axonal outgrowth. The transplantation of Schwann cells and OEG allows for spatial control of growth factors and other proteins, which are secreted by the cells.

2.3.1. Schwann Cells

Although Schwann cells originate and have predominantly been transplanted in the PNS to aid in regeneration, these cells have been shown to promote axonal outgrowth in the CNS. In studies that transected rat spinal cords and then implanted grafts containing Schwann cells and Matrigel, it was demonstrated that the number of myelinated and unmyelinated axons was greater compared to grafts containing only Matrigel and the myelinated axons formed fascicles through the conduits (Xu et al. 1997;

Xu et al. 1999). In another study transplanted Schwann cells, modified to release increased amounts of NGF, resulted in significantly more axons growing into the graft compared to treatment with Schwann cells that were not modified to release higher levels of NGF (Weidner et al. 1999). It was also demonstrated that these Schwann cells expressed the same phenotype and myelinated axons in the CNS as in the PNS. The combination of NGF and Schwann cells allows for the outgrowth of axons into the grafts due to the presence of NGF and then the Schwann cells provide direction for axonal growth due to the Bunger bands (Weidner et al. 1999). As was mentioned previously, cAMP has been investigated to promote axonal regeneration. In a study, cAMP and Schwann cells were both inserted into the spinal cord to observe whether there was a synergistic effect (Pearse et al. 2004). The results demonstrated that by implanting Schwann cells and elevating cAMP, the number of myelinated axons increased, and functional recovery was observed compared to the transplantation of only Schwann cells.

2.3.2. Olfactory Ensheathing Glia

Unlike Schwann cells, which can be transplanted in both the PNS and CNS, OEG are primarily transplanted in the CNS to promote axonal regeneration. OEG ensheath olfactory axons and shield the axons from inhibitory molecules exposed in the environment, thus allowing the axons to regenerate throughout adult life (Santos-Benito and Ramon-Cueto 2003). OEG demonstrates a promising method to ensheath the axons in other areas of the CNS that are injured and aid in regeneration. The olfactory bulb is the main supplier for OEG, and one of the main benefits of using this source for OEG is

that the glia can migrate into other regions of the CNS and integrate with other CNS glia (Santos-Benito and Ramon-Cueto 2003).

Comparisons have been made between Schwann cells and OEG for their effectiveness in promoting axonal regeneration in the CNS. In a study that compared the response of astrocytes and CSPG expression after OEG or Schwann cell transplantation in the CNS, it was demonstrated that OEG elicited less of an astrocytic response and lower expression of CSPG compared to Schwann cells (Lakatos et al. 2003). Although OEG do not induce as severe a response as Schwann cells, Schwann cells have shown more promising results in improving locomotor performance compared to OEG after adult rats have suffered from contused thoracic SCI (Takami et al. 2002).

2.3.3. Macrophages

Macrophages along with reactive microglia, as mentioned previously, are part of the initial cellular response after SCI. Macrophages are another type of cell that is transplanted in hopes to improve axonal regeneration and function within the injury site. The role of macrophages and its benefits towards axonal regeneration is controversial. Macrophages are responsible for secreting cytotoxic chemicals which enhance secondary injury thus making axonal outgrowth difficult, as well as activating astrocytes, which release factors that include CSPGs. On the other hand, these cells are also responsible for clearing myelin debris containing inhibitory molecules. This controversy has not hindered the investigation of macrophages, and currently there are studies that are in Phase I clinical trials.

2.3.4. Stem Cells and Neural/Glia Progenitor Cells

Injury to the spinal cord results in loss of neurons and axons. Although loss of neurons in the thoracic region does not contribute to major functional loss, it has detrimental consequences in the cervical and lumbar regions. Therefore, the use of embryonic/fetal stem cells and adult stem cells is highly appealing to replenish the loss of neurons and/or oligodendrocytes and for neurotrophic factor production. There are many benefits for using embryonic stem cells, such as: indefinite replication, thus never entering the senescence phase; and dividing into genetically stable cells; which can easily be genetically manipulated pre-implantation (McDonald et al. 2004; Myckatyn et al. 2004). However, the use of fetal tissue is extremely controversial, not only due to political and ethical issues, but because of the number of fetuses that are required to treat one patient, which is 10-15 (Guo et al. 2007). In a study, where human fetal neural stem cells were grafted into a lesion of a spinal cord after an induced contusion injury, it was shown that the stem cells differentiated into neurons and glia, without the formation of a tumor, and functional improvement occurred (Tarasenko et al. 2007).

The difficulty of stems cells differentiating mostly into glial cells rather than neurons has encouraged the use of progenitor cells, such as radial glial cells. These cells are intermediaries before they have fully differentiated into neurons or glia. Radial glial cells have also been transplanted after SCI. Radial glial cells were first thought to be support cells during development for new neurons (Fricker-Gates 2006). However, recent studies have shown that these cells can differentiate into neurons. Speculations on the role of the radial glial cells after development have led to suggestions that these cells might become adult neural stem cells (Doetsch 2003) and can differentiate into neurons

(Fricker-Gates 2006). The transplantation of embryonic radial glial cells after SCI has shown improved functional recovery, as well as decreased migration of activated macrophages and accumulation of CSPGs (Grumet et al. 1993). Oligodendrocyte progenitor cells have been another type of progenitor cell transplanted after SCI with the intention to remyelinate the axons and improve motor function (Keirstead et al. 2005).

Not only have these cells been transplanted alone, but cells have also been co-transplanted to improve axonal regeneration after injury. There has been a study that co-transplanted neural stem cells with Schwann cells, genetically modified to secrete NT-3, hypothesizing that the secreted NT-3 would encourage the neural stem cells to differentiate into neurons. An increase in the number of neuron-like cells was observed compared to the controls that did not have modified Schwann cells co-transplanted (Guo et al. 2007).

2.3.5. Astrocytes

It was mentioned earlier that astrocytes can also be used as a substrate for axonal outgrowth. These studies were performed *in vitro*. It was demonstrated that uniformly orienting the astrocytes and organizing the ECM and cell adhesion molecules in order to culture neurons on the astrocytes lead to the enhancement of neurites extending in a direction parallel to the astrocytes (Biran et al. 2003). The use of glial cells, such as astrocytes, as a substrate can be combined with a biomatrix to enhance neurite extension in a specific direction (Deumens et al. 2004). Glial cells were cultured on the biodegradable poly(D,L)-lactide matrices to orient the cells in a specific direction. Although this substrate did not enhance either the number of extended neurites or the

length of the neurites, the cultured cortical neurons extended neurites along the orientation of the glial cells/biomatrix substrate.

2.4. GROWTH PERMISSIVE SUBSTRATES TO ACTIVELY SUPPORT GROWING AXONS

An important strategy used to promote axonal regeneration is to provide substrates for the outgrowth to occur. Substrates that have been investigated the most to provide an adequate scaffold in the spinal cord are hydrogels and fibers, as well as transplanted cells, which were discussed earlier. Proteins and oligopeptides are coupled onto the substrates to provide a more permissive surface that mimics the ECM for the axons to anchor and extend through the nerve gap. Collagen and laminin (LN) are the more common proteins coupled to the substrates, as well as the oligopeptides, RGD, YIGSR, and IKVAV. The contribution of these proteins and oligopeptides will be discussed further later in this chapter. Transplanting cellular substrates is another approach to encourage axonal regeneration. Schwann cells, olfactory ensheathing glia (OEG), and astrocytes are examples of the cells that could be transplanted in the nerve gaps in the CNS. These cells secrete proteins and growth factors that make the microenvironment less inhibitory for axonal outgrowth.

In order for the three strategies to promote nerve regeneration, proteins must be controlled spatially and temporally in the CNS. Typically, biomaterial scaffolds are used for the spatial control of proteins in three-dimensions (3D). An important factor when developing scaffolds is that they must mimic the ECM in order to encourage axons to grow through the glial scar. Therefore, in order to control the location of the proteins,

three-dimensional scaffolds, such as hydrogels and fibers can be utilized to promote axonal regeneration in the nervous system.

2.4.1. Spatial Control: Permissive Bioactive Hydrogel Scaffolds for Enhanced Regeneration

The use of hydrogels provides a substrate for axonal outgrowth. Hydrogels are polymers that swell with the addition of water and are crosslinked. There are three main biomaterials belonging to the hydrogel family that have been used to provide a scaffold for axonal regeneration: (1) agarose, (2) alginate, and (3) collagen.

2.4.1.1. Agarose as a Scaffolding Material

Agarose, which is a thermoreversible copolymer of 1,4-linked 3,6-anhydro-α-L-galactose and 1,3-linked β-galactose, is derived from red algae. Agarose is a beneficial biomaterial to use as a scaffold for a few reasons. The hydrogel is biocompatible as it causes no adverse reaction when implanted *in vivo*. Its porosity and mechanical properties can be manipulated and optimized to maximize axonal growth (Balgude et al. 2001; Bellamkonda et al. 1995b). Most of all, agarose is beneficial because it can be used to control proteins spatially by binding proteins to the agarose and it can be used to support cell migration (Bellamkonda et al. 1995a; Borkenhagen et al. 1998).

Agarose gel can be used to encourage axonal outgrowth by covalently coupling growth promoting molecules to the agarose hydrogel, which would embody the characteristics of the ECM allowing axonal outgrowth into the glial scar and reconnect with the distal nerve. *In vitro* studies have shown that covalently coupling a growth

promoting ECM molecule, such as LN, to the agarose gel encouraged neurite outgrowth compared to a scaffold that did not have any modifications (Bellamkonda et al. 1995a; Yu et al. 1999). Along with coupling whole proteins, such as LN and collagen, oligopeptides can be bound to hydrogels as well. The oligopeptides of interest are the ones that influence cell-matrix interactions, such as RGD, which is responsible for the interaction between fibronectin and an integrin receptor, and YIGSR, which is a peptide on the β1 chain of laminin aiding in cell attachment (Borkenhagen et al. 1998).

The application of the engineered scaffold, which was a polysulfone tube containing LN-bound agarose and a slow release system of NGF, *in vivo* in the peripheral nervous system demonstrated that the regenerated myelinated axons were comparable to the regeneration found in autografts (Yu and Bellamkonda 2003).

There are different types of crosslinkers that can be used to couple the proteins to the gel. There are thermochemical bifunctional crosslinkers, such as 1,1'-carbonyldiimidazole (CDI), which can couple the protein to the agarose. Another class of crosslinkers that can be used is photocrosslinkers. These photocrosslinkers are activated by shining UV light onto the agarose gel that contains the crosslinker (Luo and Shoichet 2004). Free radicals are created, which then can be bound to the protein of interest. Photocrosslinkers can be used to covalently bind macromolecules to the agarose hydrogel. Using UV light to produce free radicals is beneficial because laser beams can be used to create patterns in the hydrogel. One such application used UV laser beams to create channels through the agarose gel, encouraging the neurons to extend their neurites down the channel, providing directional cues for the neurites (Luo and Shoichet 2004). In a study, both CDI and a photocrosslinker, benzophenone, were used to couple YIGSR

to agarose hydrogel. Results from both *in vivo* and *in vitro* experiments have shown that DRG neurite outgrowth was enhanced when cultured in 0.5% agarose. Additionally, *in vivo* the number of myelinated axons was higher in the agarose coupled to the YIGSR peptide than plain agarose (Borkenhagen et al. 1998). This study also concluded that the effectiveness of the gel was not determined by the type of crosslinker used to couple the oligopeptide. Although there is not a functional difference between the types of crosslinkers, the advantage of using the photocrosslinker is the ability to pattern the hydrogel to favor the axonal growth in a specific direction.

2.4.1.2. Alginate as a Scaffolding Material

Alginate is another scaffold material, similar to agarose, which can be utilized to control proteins spatially in order to influence axonal regeneration. Alginate can be found in brown seaweed and is a copolymer formed from α-L-glucoronic acid and β-D-mannuronic acid. Alignate sponge has been investigated as a potential scaffold to promote regeneration in the CNS after the spinal cord was transected in rats. It was shown that regenerating axons infiltrated the alginate gel significantly more as compared to collagen gels (Kataoka et al. 2004). It was also suggested that the formation of glial scar could be reduced by the alginate gel due to little infiltration of connective tissue. Although in the study above it was demonstrated that the alginate scaffold could support axonal outgrowth alone, another study showed that neuronal survival was limited and that the scaffold disappeared from the cavity within 8 weeks, and was replaced by fluid. However, when fibers coated with alginate were implanted into the cavity and BDNF was delivered after injury, it was observed that there was axonal outgrowth, as well as

neuronal survival (Novikov et al. 2002). Mixed results have been published when unmodified alginate was implanted into the spinal cord cavity. However, an experiment using anisotropic capillary alginate hydrogels demonstrated that these modified scaffolds could provide directional axonal outgrowth (Prang et al. 2006).

2.4.1.3. Collagen as a Growth Permissive Scaffold for Nerve Regeneration

The third type of hydrogel applied as a scaffold for nerve regeneration is collagen, more specifically type I collagen. Collagen is found in the ECM and helps promote axonal outgrowth and cell adhesion. Comparisons among different types of gel matrices, collagen, methylcellulose, and Biomatrix showed that collagen along with methylcellulose had the best results in regenerating axons across a peripheral nerve gap (Wells et al. 1997). It has been reported that filling tubes with ECM molecules, such as collagen, LN, and fibronectin, improves axonal regeneration. The affect of collagen and LN gels that are magnetically aligned improves the distance of axonal outgrowth compared to collagen added without any alterations (Ceballos et al. 1999; Verdu et al. 2002). Collagen gels have also been inserted into lesions after dorsal transections in rat spinal cords. Although axonal regeneration did not occur through the entire lesion area, the collagen gel along with the neurotrophin encouraged outgrowth into the matrix (Houweling et al. 1998). The study also showed minimal glial scar formation, which would provide a more promoting microenvironment for axonal regeneration.

2.4.1.4. Other Hydrogel Scaffolds

There are other hydrogels that can be used as scaffolds besides the three main ones discussed above. Some of the other hydrogels are Matrigel, NeuroGel™, and Biomatrix. Matrigel is made out of a mixture of ECM proteins, such as LN and collagen. *In vivo* studies have shown that Matrigel alone is not adequate scaffold to promote axonal outgrowth (Guenard et al. 1992; Valentini et al. 1987). However, when the Matrigel is used in conjunction with Schwann Cells, axonal outgrowth is significantly noticeable. NeuroGel™ is a crosslinked copolymer hydrogel made of N-2-(hydroxypropyl) methacrylamide. When this hydrogel was inserted into the thoracic region of the spinal cord after a contusion injury, it was observed that the rats that had implanted NeuroGel™ in the lesioned cavity had an improved locomotion according to the BBB test and there was evidence of axonal fibers infiltrating the hydrogel, thereby crossing the tissue-implant interface (Woerly et al. 2001). NeuroGel™ also demonstrated the capability to hinder glial scar formation when it was implanted in the lesion of spinal cords in adult rats (Woerly et al. 2004). Biomatrix is a hydrogel, similar to Matrigel, made of ECM proteins, such as LN. However, Biomatrix does not appear to promote regeneration as well as collagen and other hydrogels (Wells et al. 1997).

2.4.2. Spatial Control: Contact Guidance (Fibers) as a Strategy to Promote Regeneration

It was previously mentioned that besides the use of hydrogels as a scaffold, fibers could also be utilized to direct axonal growth from the proximal to distal ends of the nerve. This is another strategic technique to gain spatial control of proteins using a substrate. Tubes are inserted between the nerve gaps and then the nerve ends are sutured

to the tubes with the fibers placed through the length of the tube. Due to the fibers being oriented longitudinally through the tube, they provide the orientation for axons to grow from the proximal to distal end of the gap. Poly (L-Lactide) (PLLA) is a material that is commonly used to make filaments. In an *in vitro* study, it was demonstrated that if the PLLA was coated with LN, then the neurite outgrowth was significantly greater than neurite outgrowth on uncoated PLLA surface or the poly-L-lysine coated filaments (Rangappa et al. 2000). Tubes are generally used to encapsulate the filaments and provide an environment for axonal growth along the filaments. However, in a study conducted in the PNS, collagen filaments were sutured to the proximal and distal ends of the nerve without the aid of tubes *in vivo*. The study showed that the number of myelinated axons that regenerated was greater than that found in the group that received the autograft, although the difference was not significant (Yoshii and Oka 2001). This is the only study that did not use a conduit for the filaments or any neurotrophic factors. However, the regeneration was abundant and demonstrated that perhaps these two components are not completely necessary if the proper conditions are provided for axonal growth. Another variable that needs to be considered in the application of fibers is the number of fibers that should be inserted between the nerve ends. In studies conducted by Yoshii et al., collagen filaments were sutured to the sciatic nerve ends without the aid of a tube, two thousand filaments were connected at the ends to keep them joined over a 20 mm and 30 mm gap (Yoshii and Oka 2001; Yoshii et al. 2003). The myelinated axon regeneration was comparable to the results observed with autografts for the 20 mm gap (Yoshii and Oka 2001). However, in the case of the 30 mm gap, the axonal regeneration was significantly less. These studies suggest that a large number of filaments would aid

in axonal outgrowth. However, another study, in which PLLA filaments were inserted inside silicone tubes, demonstrated that a lower packing density of filaments elicited the greatest number of myelinated axons (Ngo et al. 2003).

Although filaments are predominantly used in the PNS, studies have been performed where filaments were inserted in CNS to promote axonal outgrowth. Carbon filaments were implanted in the lesion of a fully transected rat spinal cord. The carbon filaments allowed a scaffold for axons to advance through the lesion (Khan et al. 1991). This study was taken further, where 10,000 carbon filaments were cultured with fetal tissue and implanted into the spinal cord lesion. This condition exhibited an improvement in electrical conduction through the injured axons (Liu et al. 1995). A study conducted by the same group who inserted 2000 filaments into a nerve gap in the PNS, utilized the collagen filaments to encourage axonal regeneration in the CNS after spinal cord injury (SCI) (Yoshii et al. 2003). Four thousand collagen fibers were inserted between the two nerve ends parallel to the spinal cord. It was demonstrated that the collagen fibers provided an adequate scaffold to bridge the nerve ends and allow axons to extend across the gap.

It was previously mentioned that proteins and oligopeptides could be coupled to hydrogels. A similar method was used to couple peptides to fibers that could potentially be implanted as a scaffold in the CNS. Two laminin peptides, YIGSR and IKVAV, were coupled to poly(tetrafluoroethylene) (PTFE) fibers and DRGs were cultured to observe neurite extension (Shaw and Shoichet 2003). The peptide surface modified fibers encouraged neurite outgrowth; however, the neurites could not extend along unmodified PTFE fibers. To have successful axonal regeneration using fibers as the scaffold, it is

important to either use a biomaterial that encourages fibrin matrix formation and Schwann cell infiltration or to coat the fibers with a protein that does those things. Current research has demonstrated that fibers made out of collagen, coated with proteins, such as collagen or laminin, or oligopeptides have produced the most significant axonal regeneration. Controlling proteins spatially through fiber scaffolds allows a surface for axons to adhere, as well as orient the direction of growth.

2.5. TEMPORALLY CONTROLLING THE RELEASE OF PROTEINS

As important as it is to control the proteins spatially, it is equally imperative to control the amount of protein delivered over a period of time. Regeneration over long nerve gaps requires several months. Therefore, for axonal outgrowth to occur during this time period, the microenvironment must be actively supportive over this time scale. If proteins, such as Rho GTPases and neurotrophic factors, are only administered as a single dose at the time of implantation of the scaffold, then some of the protein will be taken up intracellularly, diffuse into the surrounding tissue, and degrade. Then there will not be a therapeutic level of protein to promote axonal outgrowth over the time necessary to have complete regeneration. For example, it was concluded that after local administration of NGF into the brain, the half-life of NGF was 30 minutes (Krewson et al. 1995). Once the effective concentration for the proteins is known, then it can be delivered and sustained. Sustaining the presence of proteins at the effective concentration can be achieved through a controlled slow release delivery system. There are currently four main techniques that are being investigated for controlling protein concentration at the site of injury over time:

(1) osmotic pumps, (2) embedded microspheres, (3) lipid microtubules and (4) enzyme dependent demand-driven trophic factor release.

2.5.1. Temporal Control: Osmotic Pumps Release Protein to Encourage Axonal Outgrowth

Osmotic pumps can be used to deliver proteins, such as neurotrophic factors, to promote axonal regeneration. Osmotic pumps are mostly utilized to deliver the proteins in the CNS. There are two parts to this delivery system, one component is the infusion pump that is usually implanted under the skin on the back of the animal, and other component is the catheter that is inserted in the lesion of the nerve.

Several studies have investigated the benefits of continuous infusion of the neurotrophic factors BDNF and NT-3 after SCI. Typically, after SCI, methylprednisolone (MP) is administered to the patient. It has been demonstrated that the levels of BDNF and NT-3 decrease after the administration of MP. In a study, after treatment of MP, it was concluded that if BDNF was continuously delivered, then the rats locomotor function improved (Kim and Jahng 2004). In a study that delivered both BDNF and NT-3 over a short time period (2 weeks) and a longer time period (8 weeks), it was shown that only the rats treated with BDNF and NT-3 over the 8 week time period allowed for the survival of the rubrospinal neurons (Novikova et al. 2002). However, rubrospinal axonal regeneration was not observed. In another study that delivered either NT-3 or BDNF for 4 weeks into the spinal cord after it was crushed, the rats treated with BDNF did not exhibit any axonal regeneration. However, fiber sprouting was observed into and through the lesion in the rats that had NT-3 administered to the spinal cord lesion

(Bradbury et al. 1999). In a study that infused only BDNF for two weeks into the rat motor cortex after SCI, sprouting of corticospinal fibers was observed; however, axonal regeneration did not occur into the peripheral nerve transplant that was placed in the lesion (Hiebert et al. 2002). The constant release of neurotrophic factors using the osmotic pump appears to exhibit therapeutic results. The site of administration seems to affect the response of axonal regeneration and fiber sprouting. The only disadvantage of utilizing the osmotic pump is the different locations of its components.

It was mentioned above that osmotic pumps can be used to deliver neurotrophic factors to the CNS to modulate intra-neuronal mechanisms. Osmotic pumps have also been utilized to infuse IN-1 antibody that neutralizes NOGO-A, an isoform of NOGO that is one of the main inhibitory molecules located in the glial scar (Brosamle et al. 2000). It was observed that after 2 weeks of IN-1 delivery, regenerating fibers were observed through the lesion in the thoracic region into the lumbar region of the spinal cord. Therefore, the use of osmotic pumps can also be used to deliver proteins that can neutralize the inhibitory environment of the glial scar.

Other than the use of osmotic pumps to deliver proteins, gelfoam, an insoluble gelatin sponge, was used to deliver chondroitinase ABC into the spinal cord lesion. The animals treated with chondroitinase ABC filled gelfoam displayed axonal regeneration of the Clarke's neurons through the lesion area and it was exhibited that CSPG was digested by the chondroitinase ABC (Yick et al. 2003).

2.5.2. Temporal Control: Slow Release of Trophic Factors Using Microspheres

Microspheres, used in drug delivery applications, are being investigated to deliver protein to the CNS in order to encourage axonal outgrowth. Microspheres have an advantage over osmotic pumps because only a single administration is needed to release the protein over time. The size of the microspheres depends upon the application. The size of the microparticles in the studies that use microspheres to promote axonal outgrowth is typically around 12-16 μm. The materials that are used to make the microsphere are typically biodegradable polymers. The use of copolymers and altering the ratio of the polymers can affect the biodegradation profiles because the polymeric characteristics, such as glass transition temperature and hydrophilicities, change (Sinha and Trehan 2003). The polymeric materials mostly used for the microspheres are poly(lactic acid) (PLA), the copolymer poly(lactic-co-glycolic acid) (PLGA) and polyphosoesters. When investigating a specific polymer or another biomaterial, it is important to make sure that when the material degrades it does not denature the protein due to the possible immunogenic response it can cause, which would alter the release profile and bioactivity (Sinha and Trehan 2003).

Most of the current research focuses on delivering NGF loaded microspheres to regenerate nerves in the PNS. However, in the CNS, one of the first studies conducted using microspheres to deliver protein to the CNS was by Camarata et al. In order to combat neurodegenerative disease, microspheres loaded with NGF were inserted that could be released *in vivo* for 4 to 5 weeks (Camarata et al. 1992). In another *in vitro* study, the number of days NGF was released was increased to 91 days. Various ratio of PLGA were tested to determine the release characteristics, as well as poly(ε-

caparolactone) (PCL) (Cao and Schoichet 1999). The surface morphology of the microspheres that are loaded versus unloaded is different. The surface of protein loaded microspheres is rougher, whereas the unloaded microspheres have a smoother surface. The smaller the microsphere, the greater the surface area, thus increasing the degradation rate of the microsphere and release of the protein. The smaller the microspheres, the less of an inflammatory response will be triggered. Therefore, nanoparticles are now being utilized *in vivo* to decrease the chances of being phagocytosed by macrophages.

2.5.3. Temporal Control: Lipid Microtubules for Sustained Release of Stimulatory Trophic Factors

Another method to slowly release protein in the CNS and PNS is the use of lipid microtubules, also referred to as microcylinders. These microtubules are hollow cylinders with a diameter of 0.5 μm (Meilander et al. 2001). The length of the microtubules varies based on the time period in which the protein, DNA, or other desired molecule needs to be released. The molecule is released at the ends of the microtubules, which is the reason why the length of the microcylinders controls the release profile of the protein. In a study previously mentioned, to aid axonal regeneration in the PNS, a two-step slow release system was developed. The first step involved NGF loaded microtubules, which had a length of 40 μm, and the second step involved embedding the loaded microtubules within the agarose hydrogel (Yu and Bellamkonda 2003). The two-step release system thus consisted first of the diffusion of the NGF from the microtubules into the agarose and then the release of the NGF from the agarose in to the gap between the two nerve ends. This slow release system allows the NGF to last longer in the nerve

gap and prevents degradation or dilution by macrophages and other fluids. Two months post-implantation, a cable formed, the number of myelinated axons was statistically similar to the autograft condition, and the density of myelinated axons was similar to that of the autograft and a normal sciatic nerve. We have shown that an agarose scaffold, containing lipid microtubules loaded with BDNF, injected into a spinal cord cavity reduced the inflammatory response and aided in axonal infiltration into the scaffold (Jain et al. 2006).

2.5.4. Temporal Control: Demand Driven Release of Trophic Factors

Another form of controlled release of a protein is the fibrin matrix, which was initially developed for wound healing. Cells that migrate to the area degrade the matrix through proteolysis, thereby releasing the contained protein (Sakiyama-Elbert and Hubbell 2000b). A fibrin matrix covalently coupled to heparin that interacted with neurotrophins, NGF, BDNF, and NT-3 was developed. It was demonstrated *in vitro* that the neurite outgrowth was enhanced when the neurotrophins were released using this delivery system as compared to when soluble neurotrophins were added to the fibrin matrix (Sakiyama-Elbert and Hubbell 2000a). When the heparin immobilized fibrin matrix was implanted in a nerve gap in the PNS, fiber sprouting was observed through the conduit to the distal end (Lee et al. 2003).

2.6. TRANSDUCTION OF PROTEINS OR TRANSGENES

2.6.1. Peptide, Non-viral Delivery Systems

The hydrophobic nature of the plasma membrane consisting of phospholipids makes it difficult for hydrophilic molecules to cross the membrane without aid. There are techniques to have cell expression or knock-down of a certain protein, such as lipids, electroporation and viral vectors. However, there were constraints to using these methods, such as lack of delivery to non-dividing cells (i.e. neurons), the optimization required for each cell type, low transfection levels, and cellular toxicity (Green et al. 2003; Zelphati and Szoka 1996). Therefore, other methods have been developed to effectively transfer protein. To transduce proteins into the cells, transduction peptides have been identified and utilized. These peptides are cell-permeable peptides, also referred to as protein transduction domains (PTDs). PTDs have been identified to allow the internalization of larger proteins (Joliot and Prochiantz 2004). These transduction peptides can be naturally found, such as the Tat peptide; synthetically formulated, Pep-1 for example; or created by phage display, such as Pep-7 (Joliot and Prochiantz 2004).

Tat is a sequence of peptides that can be found on the human immunodeficiency virus (HIV-1). It is typically 8-11 amino acids in length and does not contain any genetic viral component. One of the theories as to the internalization of the peptide, as well as the compound is through ionic interactions. The interactions between the peptide and the anionic structures on the cell surface then give way to the endocytic internalization of Tat and desired compound (Vives 2003). Once internalized, the amino acids that make up the Tat peptide sequence are catabolized (Vives 2003). In order to deliver the desired compound, it must be conjugated to the Tat peptide and then replicated. This custom

delivery can be a tedious process. There are several studies that have conjugated proteins to the Tat peptide to be delivered within the lesion site after SCI (Monnier et al. 2003; Winton et al. 2002). In our study, we have also used Rho GTPases that have been conjugated to Tat. Tat has also been conjugated to siRNA, which shows promise in knocking-out the inhibitory molecules, such as CSPGs.

There are commercially available generic peptides, such as Pep-1 also known as Chariot. Pep-1 is a 21 residue peptide, which is lysine-rich, that does not require pre-chemical covalent coupling (Deshayes et al. 2004; Gros et al. 2006; Morris et al. 2001). Multiple peptide strands form complexes with the protein, peptide, or antibody in question. The Pep-1 sequences are on the outside of the complex with the protein on the inside, so that Pep-1 interacts with the cellular membrane when it inserts itself as a transmembrane pore structure. Once the protein has transduced across the cellular membrane, the complex between the peptide and the compound is broken (Deshayes et al. 2004). In an *in vitro* study it was shown that this peptide aided in the transduction of Rho GTPases, CA-Cdc42 and CA-Rac1, without inducing cytotoxic effects or disturbing the conformation of the proteins, allowing the proteins to continue with their function (Jain et al. 2004). The McKerracher laboratory has also developed a peptide construct that is conjugated to proteins for transduction across cellular membranes (Winton et al. 2002).

2.6.2. Viral-based Delivery Systems

Viral vectors have been used to induce the production of proteins by the endogenous local cells. There are four viral vectors that are used for gene delivery: (1)

adenoviral vectors, (2) adeno-associated vectors (AAV), (3) retroviral vectors, (4) lentiviral vectors, and (5) Herpes simplex viral vectors (HSV). These five viruses can be distinguished into two categories, those that integrate their genomes into the host cellular chromatin or viruses that are in the cell nucleus as extrachromosomal episomes (Thomas et al. 2003). The target cells for gene therapy are one of the determining factors as to which viral vector should be used. For efficient transfection of non-dividing cells, the use of viruses that integrate their genomes into the host cellular chromatin are utilized, such as lentiviruses. However, for cells that proliferate, integrating vectors are the best choice to allow for stable genetic alterations (Thomas et al. 2003).

Adenoviral vectors consist of non-enveloped DNA. This viral vector is advantageous because they can infect a wide variety of cell types, which include non-dividing cells, and can be grown to high titers (Robbins et al. 1998). However, the advenoviruses induce a strong immune response. Adenoviral vectors were being used in clinical trials in the 1990s. However, the use of adenoviral vectors was reconsidered after a tragic case; a patient suffered from a massive inflammatory response when the adenoviral vector, which was locally delivered, systemically traveled, leading to multi-organ failure (Thomas et al. 2003). This case and other studies lead to the re-evaluation into the use of viral vectors. Newer generations of adenoviral vectors have been developed, which do not elicit as severe an immunogenic response and has shown to be effective in animal models (Thomas et al. 2003). Although the newer generations of the adenoviruses have elicited less of an inflammatory response, the CNS studies that have used this vector have shown that the toxicity is still an issue.

The second type of viral vector, AAV, is a member of the parvovirus family. The benefits are that they infect non-dividing cells and the integration location into the chromosome is known each time. However, the disadvantages are that this vector requires a helper virus, such as an adenovirus or HSV, for replication and the infection efficiency is low. After SCI, AAVs encoding for either BDNF or NT-3 were transfected into neurons distal to the lesion site to encourage axonal infiltration into the caudal spinal cord. It was observed that although the neurons did express BDNF or NT-3 for 16 weeks post-injection, the axons did not infiltrate the caudal spinal cord segment (Blits et al. 2003).

Unlike the adenoviral vectors, the retrovirus is made up of an enveloped RNA virus. The benefits of using the retroviral vector are that it can stably infect dividing cells, and has high infection efficiency (Robbins et al. 1998). The disadvantage of using this viral vector is that it does not have the capacity to deliver large genes, the cells must be dividing, and it is difficult to concentrate and purify the vector (Blits and Bunge 2006; Robbins et al. 1998). The use of retroviruses after SCI is limited due to the fact that these vectors cannot infect non-dividing cells, such as neurons.

The benefits of the lentiviral vectors are that these vectors are able to transfect both proliferating and non-dividing cells. Therefore, in the CNS it allows for the transduction of genetic material in both glia and neurons (Blits and Bunge 2006). The transgene using the lentiviral vector can be around 8-10 kb in size (Azzouz et al. 2004). A limited inflammatory response is seen after use of this vector. The fifth viral vector, HSV, can also infect non-dividing cells, however, the expression is transient.

In a study that compared the efficiency and protein expression after delivery of a transgene into spinal cord using lentiviral, adenoviral, and retroviral vectors, it was shown that the protein expression lasted longer when the lentiviral vector was used compared to the other two vectors, over 4 weeks (Abdellatif et al. 2006). For transient delivery, the retroviral vector can be useful, which by 2 weeks showed that protein expression had decreased. However, cellular expression of the protein at 2 weeks when the adenoviral vector was used to deliver the transgene, demonstrating that this vector would be useful if protein expression is desired for a couple of weeks.

2.7. SPINAL CORD INJURY MODELS

The implementation of the strategies mentioned in this chapter requires a SCI model that allows for full behavioral and immunohistochemical analysis. There are four main SCI models that are relevant to examine axonal outgrowth after injury. These include a contusion model, a complete transection model, a lateral hemisection model, and a dorsal over-hemisection model, each with its own advantages and disadvantages.

2.7.1. Contusion Injury

The contusion model is the most clinically relevant, since the vast majority of SCI in humans results from a “fracture-dislocation” of a particular vertebrae and resultant compression of the spinal cord running through the spinal canal of each vertebra. In humans and rats, this injury results in a significant amount of cell death and tissue damage leading to the formation of a fluid filled cyst within the center of the spinal cord surrounded by a rim of intact tissue. Cyst formation takes place over the course of weeks

and the amount of preserved tissue is highly variable (Kwon et al. 2002). A disadvantage of this model is the axonal sparing that occurs after injury (Steward et al. 2003). It is difficult to distinguish between regenerated axons versus axons that were spared at the time of injury. Therefore, in a study where the main focus is axonal outgrowth, this model has its limitations.

2.7.2. Complete Transection Injury

In place of the contusion injury, many labs have utilized complete transection models to examine regeneration of specific axon pathways. However, while this model eliminates some of the overlap between sprouting and regenerating fibers, it is a drastic model that completely eliminates function below the injury site, which is stressful on the animal.

2.7.3. Lateral Hemisection Injury

In the lateral hemisection model, all pathways on one side of the spinal cord are severed. Therefore, this model does not raise questions as to whether the axons are spared or regenerated. Also, this allows for the examination of multiple pathways. However, the drawback to this model is that the intact axons from the non-lesioned side of the spinal cord may sprout into dennervated areas below the lesion (Kwon et al. 2002).

2.7.4. Dorsal Over-hemisection Injury

In the dorsal over-hemisection model, damage is limited to the dorsal columns and the dorsal CST bilaterally. Although the chance of spared axons is slim due to the

bilateral transaction of the CST, it will be possible to distinguish between spared and regenerated axons by injecting BDA into the sensory motor cortex where these axons originate and examining the distribution of BDA-positive axons in the distal spinal cord.

We have chosen to use the dorsal over-hemisection model because the CST is critically important in humans but has remained generally refractory to all attempts at promoting its regeneration. Consequently, the dorsal CST will give us the most sensitive and potentially clinically important read-out of regeneration in response to our different and combined therapeutic approach. The damage is limited to the dorsal columns; therefore, the animals regain the ability to urinate and defecate, thereby requiring less care and experiencing less mortality than animals with larger spinal cord transections.

2.8. CONCLUSIONS

Many recent advancements in CNS regeneration have been due to the utilization of nano- and micro-technologies. Most of the technology that has been developed has been geared towards controlling proteins spatially and temporally. There are three main strategies used to elicit axonal outgrowth after injury that allow spatial and temporal control of proteins. These are 1) providing permissive bioactive substrates for the axonal outgrowth, (2) using trophic factors to stimulate growth, and (3) alleviating inhibitory signals present in the extracellular environment to allow axons to regenerate between the proximal and distal ends.

This chapter briefly summarizes the use of proteins, such as Rho GTPases and chABC in the extrinsic and intrinsic strategies. To deliver these proteins, strategies involving spatial and temporal control have been developed. Cells, hydrogel scaffolds,

and fibers have been implanted within the cavity for spatial control. For temporal control, osmotic pumps, microspheres, lipid microtubules, and demand driven release of trophic factors have been used. Previously, most of these strategies have been used alone, but recent studies have been using them in combination to enhance axonal outgrowth. The key combination, however, remains elusive and is the focus of active ongoing investigation.

2.9. REFERENCES

Abdellatif AA, Pelt JL, Benton RL, Howard RM, Tsoulfas P, Ping P, Xu XM, Whittemore SR. 2006. Gene delivery to the spinal cord: comparison between lentiviral, adenoviral, and retroviral vector delivery systems. J Neurosci Res 84(3):553-567.

Aguayo AJ, David S, Bray GM. 1981. Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. The Journal of experimental biology 95:231-240.

Azzouz M, Kingsman SM, Mazarakis ND. 2004. Lentiviral vectors for treating and modeling human CNS disorders. The journal of gene medicine 6(9):951-962.

Balgude AP, Yu X, Szymanski A, Bellamkonda RV. 2001. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. Biomaterials 22(10):1077-1084.

Bandtlow CE. 2003. Regeneration in the central nervous system. Exp Gerontol 38(1-2):79-86.

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(6):1375-1381.

Bellamkonda R, Ranieri JP, Aebischer P. 1995a. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension in vitro. J Neurosci Res 41(4):501-509.

Bellamkonda R, Ranieri JP, Bouche N, Aebischer P. 1995b. Hydrogel-based three-dimensional matrix for neural cells. J Biomed Mater Res 29(5):663-671.

Biran R, Noble MD, Tresco PA. 2003. Directed nerve outgrowth is enhanced by engineered glial substrates. Exp Neurol 184(1):141-152.

Blits B, Bunge MB. 2006. Direct gene therapy for repair of the spinal cord. J Neurotrauma 23(3-4):508-520.

Blits B, Oudega M, Boer GJ, Bartlett Bunge M, Verhaagen J. 2003. Adeno-associated viral vector-mediated neurotrophin gene transfer in the injured adult rat spinal cord improves hind-limb function. Neuroscience 118(1):271-281.

Borkenhagen M, Clemence JF, Sigrist H, Aebischer P. 1998. Three-dimensional extracellular matrix engineering in the nervous system. J Biomed Mater Res 40(3):392-400.

Bradbury EJ, Khemani S, Von R, King, Priestley JV, McMahon SB. 1999. NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. Eur J Neurosci 11(11):3873-3883.

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(6881):636-640.

Brosamle C, Huber AB, Fiedler M, Skerra A, Schwab ME. 2000. Regeneration of lesioned corticospinal tract fibers in the adult rat induced by a recombinant, humanized IN-1 antibody fragment. J Neurosci 20(21):8061-8068.

Cafferty WB, Yang SH, Duffy PJ, Li S, Strittmatter SM. 2007. Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans. J Neurosci 27(9):2176-2185.

Camarata PJ, Suryanarayanan R, Turner DA, Parker RG, Ebner TJ. 1992. Sustained release of nerve growth factor from biodegradable polymer microspheres. Neurosurgery 30(3):313-319.

Cao X, Schoichet MS. 1999. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. Biomaterials 20(4):329-339.

Ceballos D, Navarro X, Dubey N, Wendelschafer-Crabb G, Kennedy WR, Tranquillo RT. 1999. Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration. Exp Neurol 158(2):290-300.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81(7):1137-1146.

David S, Lacroix S. 2003. Molecular Approaches to Spinal Cord Repair. Annu Rev Neurosci.

Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F. 2004. Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. Biochemistry 43(6):1449-1457.

Deumens R, Koopmans GC, Den Bakker CG, Maquet V, Blacher S, Honig WM, Jerome R, Pirard JP, Steinbusch HW, Joosten EA. 2004. Alignment of glial cells stimulates directional neurite growth of CNS neurons in vitro. Neuroscience 125(3):591-604.

Doetsch F. 2003. The glial identity of neural stem cells. Nat Neurosci 6(11):1127-1134.

Dubreuil CI, Winton MJ, McKerracher L. 2003. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. J Cell Biol 162(2):233-243.

Fawcett JW, Asher RA. 1999. The glial scar and central nervous system repair. Brain Res Bull 49(6):377-391.

Fricker-Gates RA. 2006. Radial glia: a changing role in the central nervous system. Neuroreport 17(11):1081-1084.

GrandPre T, Li S, Strittmatter SM. 2002. Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417(6888):547-551.

Green I, Christison R, Voyce CJ, Bundell KR, Lindsay MA. 2003. Protein transduction domains: are they delivering? Trends Pharmacol Sci 24(5):213-215.

Gros E, Deshayes S, Morris MC, Aldrian-Herrada G, Depollier J, Heitz F, Divita G. 2006. A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction. Biochimica et biophysica acta 1758(3):384-393.

Grumet M, Flaccus A, Margolis RU. 1993. Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules. J Cell Biol 120(3):815-824.

Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P. 1992. Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. J Neurosci 12(9):3310-3320.

Guo JS, Zeng YS, Li HB, Huang WL, Liu RY, Li XB, Ding Y, Wu LZ, Cai DZ. 2007. Cotransplant of neural stem cells and NT-3 gene modified Schwann cells promote the recovery of transected spinal cord injury. Spinal Cord 45(1):15-24.

Hennigan A, O'Callaghan RM, Kelly AM. 2007. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochemical Society transactions 35(Pt 2):424-427.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. 2002. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J Neurosci Res 69(2):160-168.

Hoke A, Silver J. 1996. Proteoglycans and other repulsive molecules in glial boundaries during development and regeneration of the nervous system. Prog Brain Res 108:149-163.

Houweling DA, Lankhorst AJ, Gispen WH, Bar PR, Joosten EA. 1998. Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. Exp Neurol 153(1):49-59.

Jain A, Brady-Kalnay SM, Bellamkonda RV. 2004. Modulation of Rho GTPase activity alleviates chondroitin sulfate proteoglycan-dependent inhibition of neurite extension. J Neurosci Res 77(2):299-307.

Jain A, Kim YT, McKeon RJ, Bellamkonda RV. 2006. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. Biomaterials 27(3):497-504.

Joliot A, Prochiantz A. 2004. Transduction peptides: from technology to physiology. Nat Cell Biol 6(3):189-196.

Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J Neurosci 22(7):2792-2803.

Kataoka K, Suzuki Y, Kitada M, Hashimoto T, Chou H, Bai H, Ohta M, Wu S, Suzuki K, Ide C. 2004. Alginate enhances elongation of early regenerating axons in spinal cord of young rats. Tissue Eng 10(3-4):493-504.

Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O. 2005. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25(19):4694-4705.

Khan T, Dauzvardis M, Sayers S. 1991. Carbon filament implants promote axonal growth across the transected rat spinal cord. Brain Res 541(1):139-145.

Kim DH, Jahng TA. 2004. Continuous brain-derived neurotrophic factor (BDNF) infusion after methylprednisolone treatment in severe spinal cord injury. J Korean Med Sci 19(1):113-122.

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(6):1566-1569.

Kozma R, Ahmed S, Best A, Lim L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15(4):1942-1952.

Krewson CE, Klarman ML, Saltzman WM. 1995. Distribution of nerve growth factor following direct delivery to brain interstitium. Brain Res 680(1-2):196-206.

Kwon BK, Oxland TR, Tetzlaff W. 2002. Animal models used in spinal cord regeneration research. Spine 27(14):1504-1510.

Lakatos A, Barnett SC, Franklin RJ. 2003. Olfactory ensheathing cells induce less host astrocyte response and chondroitin sulphate proteoglycan expression than Schwann cells following transplantation into adult CNS white matter. Exp Neurol 184(1):237-246.

Lee AC, Yu VM, Lowe JB, 3rd, Brenner MJ, Hunter DA, Mackinnon SE, Sakiyama-Elbert SE. 2003. Controlled release of nerve growth factor enhances sciatic nerve regeneration. Exp Neurol 184(1):295-303.

Levi-Montalcini R. 1987. The nerve growth factor 35 years later. Science 237(4819):1154-1162.

Li S, Strittmatter SM. 2003. Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. J Neurosci 23(10):4219-4227.

Liu LS, Khan T, Sayers ST, Dauzvardis MF, Trausch CL. 1995. Electrophysiological improvement after co-implantation of carbon filaments and fetal tissue in the contused rat spinal cord. Neurosci Lett 200(3):199-202.

Luo Y, Shoichet MS. 2004. A photolabile hydrogel for guided three-dimensional cell growth and migration. Nat Mater 3(4):249-253.

Mattson MP, Taylor-Hunter A, Kater SB. 1988. Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. J Neurosci 8(5):1704-1711.

McDonald JW, Becker D, Holekamp TF, Howard M, Liu S, Lu A, Lu J, Platik MM, Qu Y, Stewart T, Vadivelu S. 2004. Repair of the injured spinal cord and the potential of embryonic stem cell transplantation. J Neurotrauma 21(4):383-393.

McKeon RJ, Hoke A, Silver J. 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136(1):32-43.

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(4):805-811.

Meilander NJ, Yu X, Ziats NP, Bellamkonda RV. 2001. Lipid-based microtubular drug delivery vehicles. J Control Release 71(1):141-152.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22(3):319-330.

Morgenstern DA, Asher RA, Fawcett JW. 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 137:313-332.

Morris MC, Depollier J, Mery J, Heitz F, Divita G. 2001. A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19(12):1173-1176.

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(3):757-767.

Myckatyn TM, Mackinnon SE, McDonald JW. 2004. Stem cell transplantation and other novel techniques for promoting recovery from spinal cord injury. Transplant immunology 12(3-4):343-358.

Ngo TT, Waggoner PJ, Romero AA, Nelson KD, Eberhart RC, Smith GM. 2003. Poly(L-Lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions. J Neurosci Res 72(2):227-238.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE. 2002. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22(23):10368-10376.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Novikov LN, Novikova LN, Mosahebi A, Wiberg M, Terenghi G, Kellerth JO. 2002. A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury. Biomaterials 23(16):3369-3376.

Novikova LN, Novikov LN, Kellerth JO. 2002. Differential effects of neurotrophins on neuronal survival and axonal regeneration after spinal cord injury in adult rats. J Comp Neurol 452(3):255-263.

Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB. 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10(6):610-616.

Prang P, Muller R, Eljaouhari A, Heckmann K, Kunz W, Weber T, Faber C, Vroemen M, Bogdahn U, Weidner N. 2006. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 27(19):3560-3569.

Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT. 2002. Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34(6):895-903.

Rangappa N, Romero A, Nelson KD, Eberhart RC, Smith GM. 2000. Laminin-coated poly(L-lactide) filaments induce robust neurite growth while providing directional orientation. J Biomed Mater Res 51(4):625-634.

Robbins PD, Tahara H, Ghivizzani SC. 1998. Viral vectors for gene therapy. Trends in biotechnology 16(1):35-40.

Sakiyama-Elbert SE, Hubbell JA. 2000a. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Control Release 69(1):149-158.

Sakiyama-Elbert SE, Hubbell JA. 2000b. Development of fibrin derivatives for controlled release of heparin-binding growth factors. J Control Release 65(3):389-402.

Santos-Benito FF, Ramon-Cueto A. 2003. Olfactory ensheathing glia transplantation: a therapy to promote repair in the mammalian central nervous system. Anat Rec 271B(1):77-85.

Schweigreiter R, Bandtlow CE. 2006. Nogo in the injured spinal cord. J Neurotrauma 23(3-4):384-396.

Shaw D, Shoichet MS. 2003. Toward spinal cord injury repair strategies: peptide surface modification of expanded poly(tetrafluoroethylene) fibers for guided neurite outgrowth in vitro. J Craniofac Surg 14(3):308-316.

Sinha VR, Trehan A. 2003. Biodegradable microspheres for protein delivery. J Control Release 90(3):261-280.

Steward O, Zheng B, Tessier-Lavigne M. 2003. False resurrections: distinguishing regenerated from spared axons in the injured central nervous system. J Comp Neurol 459(1):1-8.

Takami T, Oudega M, Bates ML, Wood PM, Kleitman N, Bunge MB. 2002. Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. J Neurosci 22(15):6670-6681.

Tarasenko YI, Gao J, Nie L, Johnson KM, Grady JJ, Hulsebosch CE, McAdoo DJ, Wu P. 2007. Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. J Neurosci Res 85(1):47-57.

Thomas CE, Ehrhardt A, Kay MA. 2003. Progress and problems with the use of viral vectors for gene therapy. Nature reviews 4(5):346-358.

Valentini RF, Aebischer P, Winn SR, Galletti PM. 1987. Collagen- and laminin-containing gels impede peripheral nerve regeneration through semipermeable nerve guidance channels. Exp Neurol 98(2):350-356.

Verdu E, Labrador RO, Rodriguez FJ, Ceballos D, Fores J, Navarro X. 2002. Alignment of collagen and laminin-containing gels improve nerve regeneration within silicone tubes. Restor Neurol Neurosci 20(5):169-179.

Vives E. 2003. Cellular uptake [correction of utake] of the Tat peptide: an endocytosis mechanism following ionic interactions. J Mol Recognit 16(5):265-271.

Vourc'h P, Moreau T, Arbion F, Marouillat-Vedrine S, Muh JP, Andres C. 2003. Oligodendrocyte myelin glycoprotein growth inhibition function requires its conserved leucine-rich repeat domain, not its glycosylphosphatidyl-inositol anchor. J Neurochem 85(4):889-897.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. 2002a. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420(6911):74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z. 2002b. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417(6892):941-944.

Weidner N, Blesch A, Grill RJ, Tuszynski MH. 1999. Nerve growth factor-hypersecreting Schwann cell grafts augment and guide spinal cord axonal growth and remyelinate central nervous system axons in a phenotypically appropriate manner that correlates with expression of L1. J Comp Neurol 413(4):495-506.

Wells MR, Kraus K, Batter DK, Blunt DG, Weremowitz J, Lynch SE, Antoniades HN, Hansson HA. 1997. Gel matrix vehicles for growth factor application in nerve gap injuries repaired with tubes: a comparison of biomatrix, collagen, and methylcellulose. Exp Neurol 146(2):395-402.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L. 2002. Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277(36):32820-32829.

Woerly S, Doan VD, Evans-Martin F, Paramore CG, Peduzzi JD. 2001. Spinal cord reconstruction using NeuroGel implants and functional recovery after chronic injury. J Neurosci Res 66(6):1187-1197.

Woerly S, Doan VD, Sosa N, de Vellis J, Espinosa-Jeffrey A. 2004. Prevention of gliotic scar formation by NeuroGel allows partial endogenous repair of transected cat spinal cord. J Neurosci Res 75(2):262-272.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM. 2002. A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5(12):1302-1308.

Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. 1997. Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. J Neurocytol 26(1):1-16.

Xu XM, Zhang SX, Li H, Aebischer P, Bunge MB. 1999. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisected adult rat spinal cord. Eur J Neurosci 11(5):1723-1740.

Yick LW, Cheung PT, So KF, Wu W. 2003. Axonal regeneration of Clarke's neurons beyond the spinal cord injury scar after treatment with chondroitinase ABC. Exp Neurol 182(1):160-168.

Yoshii S, Oka M. 2001. Collagen filaments as a scaffold for nerve regeneration. J Biomed Mater Res 56(3):400-405.

Yoshii S, Oka M, Shima M, Taniguchi A, Akagi M. 2003. Bridging a 30-mm nerve defect using collagen filaments. J Biomed Mater Res 67A(2):467-474.

Yu X, Bellamkonda RV. 2003. Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Eng 9(3):421-430.

Yu X, Dillon GP, Bellamkonda RB. 1999. A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. Tissue Eng 5(4):291-304.

Zelphati O, Szoka FC, Jr. 1996. Mechanism of oligonucleotide release from cationic liposomes. Proceedings of the National Academy of Sciences of the United States of America 93(21):11493-11498.

Zheng B, Atwal J, Ho C, Case L, He XL, Garcia KC, Steward O, Tessier-Lavigne M. 2005. Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proceedings of the National Academy of Sciences of the United States of America 102(4):1205-1210.

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M. 2003. Lack of enhanced spinal regeneration in Nogo-deficient mice. Neuron 38(2):213-224.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D. 1998. Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Exp Neurol 154(2):654-662.

Zuo J, Neubauer D, Graham J, Krekoski CA, Ferguson TA, Muir D. 2002. Regeneration of axons after nerve transection repair is enhanced by degradation of chondroitin sulfate proteoglycan. Exp Neurol 176(1):221-228.

CHAPTER 3

MODULATION OF RHO GTPASES TO OVERCOME CSPG-DEPENDENT INHIBITION ON NEURITE EXTENSION *IN VITRO*

(As published with S.M. Brady-Kalnay and R.V. Bellamkonda, Journal of Neuroscience Research, 77(2004) 299-307).

The central nervous system (CNS) fails to regenerate after injury. A glial scar forms at the injury site, contributing to regenerative failure partly due to the chondroitin sulfate proteoglycans (CSPGs) in the glial scar. The family of Rho GTPases, which include Cdc42, Rac1, and RhoA, is involved in growth cone dynamics. Although the response of neural cells to the inactivation of Rho when contacting myelin-related substrates, or CSPG, has been investigated, Rac1 and Cdc42's ability to modulate CSPG-dependent inhibition has yet to be explored. In this study, a stripe assay was utilized to examine the effects of modulating all three Rho GTPases on neurite extension across inhibitory CSPG lanes. Alternating laminin (LN) and CSPG lanes were created and NG108-15 cells and E9 chick dorsal root ganglions (DRGs), were cultured on the lanes. Using the protein delivery agent Chariot®, the neuronal response to exposure of constitutively active (CA) and dominant negative (DN) mutants of the Rho GTPases, along with the bacterial toxin C3, was determined by quantifying the percent ratio of neurites crossing the CSPG lanes. CA-Cdc42, CA-Rac1, and C3 transferase significantly increased the number of neurites crossing into the CSPG lanes compared to the negative controls for both the NG108-15 cells and the E9 chick DRGs. We also show that these

mutant proteins require the delivery vehicle, Chariot®, to enter the neurons and affect neurite extension. Therefore, activation of Cdc42 and Rac, as well as inhibition of Rho, helps overcome the CSPG-dependent inhibition of neurite extension.

3.1 INTRODUCTION

Physical injury to the CNS often results in permanent functional loss as astroglial scar at the site of injury results in a non-permissive environment for regeneration. Astroglial scar contains astrocytes, oligodendrocytes, oligodendrocyte precursors, meningeal cells, and microglia, that produce inhibitory molecules (Fawcett and Asher 1999) that have been implicated in regenerative failure (David and Lacroix 2003); (Hoke and Silver 1996). Inhibitory macromolecules include myelin-associated glycoproteins (MAG) (McKerracher et al. 1994; Mukhopadhyay et al. 1994), NOGO-A (Niederost et al. 2002), and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al. 2002), which are myelin-related, and CSPGs, a family of non-myelin molecules (reviewed in (Spencer et al. 2003). CSPGs, consisting of a protein core and glycosaminoglycan (GAG) side chains (referred to as chondroitin sulfate) (Morgenstern et al. 2002), are classified as aggrecan, phosphacan, neurocan, brevican, NG2, and versican (Tang 2003). CSPGs are mainly produced by astrocytes and are also expressed by oligodendrocyte precursors and meningeal cells (Fawcett and Asher 1999).

There are two possible strategies, extrinsic and intrinsic, to regenerate nerves through the glial scar. Extrinsic approaches focus on removing inhibition, typically by using the enzyme chondroitinase ABC to cleave the GAG into its constituent

disaccharides. This strategy helps partially alleviate CSPG mediated inhibition *in vitro* and *in vivo* (Bradbury et al. 2002; McKeon et al. 1995; Zuo et al. 1998).

In this study, we investigate an intrinsic strategy of alleviating the inhibitory influence of CSPGs through modulation of Rho GTPases. The Rho GTPase family, particularly Cdc42, Rac1, and RhoA, are involved in the growth cone dynamics (Hall 1998). Cdc42 induces filopodia growth and Rac1 promotes lamellipodia formation (Nobes and Hall 1995). RhoA responds to inhibitory cues by inducing growth cone collapse (Hall 1998). Rho GTPases are active when GTP-bound and are inactive when GDP-bound. Mutant forms of the Rho GTPases that mimic either the GTP-bound, constitutively active (CA), or GDP-bound, dominant negative (DN), have been constructed (Coso et al. 1995; Kozma et al. 1995). We utilized CA- and DN-glutathione S-transferase (GST)-fusion proteins of the Rho GTPases.

It has been reported previously that MAG, NOGO-A, and OMgp contribute to the CNS inhibition (reviewed in (Filbin 2003). Alleviation of myelin-related inhibition by inhibiting the activation of Rho and its downstream effector ROCK, using C3 transferase (C3) or Y27632 has been reported. Typically, these proteins are transduced into cells through trituration (Jin and Strittmatter 1997). However, this disrupts cell function and reduces cell viability. Therefore, delivery reagents that transduce the Rho GTPases while preserving cell viability are needed. Therefore TAT, a human immunodeficiency viral peptide, was fused to C3 to aid in protein uptake (Winton et al. 2002); (Dubreuil et al. 2003); (Monnier et al. 2003). While these studies have mainly focused on Rho and ROCK inactivation on both myelin and CSPG surfaces, the effect of modulating Rac1 and Cdc42 on CSPG-dependent inhibition is still unclear.

Using a generic protein delivery agent, we investigated the effect of modulating Rac1 and Cdc42, as well as C3, on CSPG-mediated inhibition (specifically aggrecan). We used a modified *in vitro* Bonhoeffer stripe assay (Vielmetter et al. 1990), consisting of alternating lanes of laminin (LN) and CSPG (Snow et al. 2002); (Chen et al. 2002);(Monnier et al. 2003). CSPG lanes are non-permissive for cellular attachment and neurite outgrowth (Sango et al. 2003; Schmalfeldt et al. 2000). Laminin was used to aid in neuronal attachment on the non-CSPG lanes (Condic and Lemons 2002). NG108-15 cells and whole E9 chick dorsal root ganglions (DRGs) were added on the lanes and transduced with Rho GTPase mutant proteins. The percent ratio of neurites that grew along the LN lanes versus the percentage of neurites that crossed into the CSPG lanes was quantified as a measure of alleviation of CSPG-dependent inhibition.

3.2 MATERIALS AND METHODS

3.2.1. Surface Modification of Tissue Culture Dishes

Alternating CSPG and LN (Invitrogen, Carlsbad, CA) lanes were created on 60 mm diameter tissue culture dishes using a modified version of the Bonhoeffer method (Vielmetter et al. 1990). The CSPG (aggrecan), a gift from Dr. Arnold Caplan's laboratory (Department of Biology, Case Western Reserve University, Cleveland, OH) was purified from embryonic chick chondrocytes. The 60 mm dishes were initially coated with nitrocellulose (5 cm^2 of nitrocellulose dissolved in 12 mL methanol) and air dried for 30 minutes. The Bonhoeffer silicone matrices (Max Planck Institute, Tubingen, Germany) were placed over a uniform nitrocellulose region. CSPG was dissolved in distilled deionized water for a final concentration of 150 μg/mL, and 25 μg/mL of bovine

albumin serum-FITC (BSA, Sigma-Aldrich, Milwaukee, WI) was added for visualization. This mixture was injected into the matrix chamber, aspirated into the channels, and allowed to bind to the nitrocellulose for 10 minutes. The CSPG solution was then aspirated out of the channels, and the procedure was repeated 4 additional times. Next, 2 % bovine serum albumin (BSA, Sigma, Milwaukee, WI) was aspirated through the channels and allowed to incubate for ten minutes to block any unbound sites. This cycle was performed 3 times. Phosphate buffered saline (PBS) was aspirated into the channels in three rapid successions to remove any unbound protein. The silicone matrix was removed and LN (40 μg/mL) was added over the modified region for 30 min. To block the unbound nitrocellulose, 2% BSA was added to the entire dish for 10 minutes. After removing the BSA, the dishes were rinsed with Dulbecco's Modified Eagle Media (DMEM, Invitrogen, Carlsbad, CA) then stored at 37°C with 2 mL of DMEM to be used within a few hours.

3.2.2. NG108-15 Cells Neurite Outgrowth Assays

NG108-15 cells (ATCC, Manassas, VA) were removed from T75 culture flasks by trypsinization and then resuspended in Neurobasal-A media (Invitrogen, Carlsbad, CA) supplemented with N-2, L-glutamine, and 1% penicillin streptomycin. The DMEM was removed from the modified tissue culture dishes, and 2 mL of Neurobasal-A media along with 2×10^4 NG108-15 cells were added over the lane region. The cultures were incubated for 4 hours in 37°C and 5% CO_2. By 4 hrs, NG108-15 cells typically formed concise lanes, adhering only to LN lanes, and not to CSPG containing lanes. The Rho GTPases: L61 Cdc42(CA)-GST, N17 Cdc42(DN)-GST, L61 Rac1(CA)-GST, N17

Rac1(DN)-GST, and L63 RhoA(CA)-GST, as well as C3 transferase (Cytoskeleton, Denver, CO), were prepared for protein transduction by complexing them with Chariot® described below. For the GST control, the GST fusion protein is complexed to the Chariot®. To form the protein transduction complexes, 8 μL of 1 mg/mL protein in 200 μL of PBS and 20 μL of Chariot® (Active Motif, Carlsbad, CA), which was dissolved previously in sterile deionized distilled water, was further diluted in 200 μL of deionized distilled water and the two solutions were mixed and incubated at room temperature for 30 min. For the C3, the molar equivalent (2 μg/mL) to the other mutant proteins was transduced. Four hours after plating the cells, 400 μL of the Neurobasal-A medium was removed from the culture and the protein/Chariot® complex was added to the dish. For the media control, sterile deionized distilled water and PBS were added to the cultures without protein or Chariot®. Five hours after plating, 1 mL of Neurobasal-A media was added for a final culture media volume of 3 mL. The cultures were placed in humidified incubators at 37°C and 5% CO_2 and were imaged under light microscopy after 48 hr as described below. The cultures were then fixed using 4% paraformaldehyde and stored for further analysis.

A dose response study was performed using the following final concentrations of CA-Cdc42, 1 μg/mL, 2 μg/mL, 3 μg/mL, 4 μg/mL, 6 μg/mL, and 8 μg/mL. The culturing, incubation, and protein transduction were performed as described above.

To evaluate whether the Chariot® aids proteins to enter the cells, protein transductions with CA-Rac1 and C3 were performed without the addition of Chariot®. The same procedures were performed as described above, however, the same solution was made without the Chariot® reagent.

3.2.3. DRG Neurite Outgrowth Assays

Sixty mm culture dishes with alternating lanes of LN and CSPG were prepared as described above. Whole E9 White Leghorn chicks were dissected and 3-4 whole DRGs were cultured on each of the modified tissue culture dishes. Neurobasal medium (500 μL) supplemented with B-27 supplement and L-glutamine was added to the culture dishes. The protein/Chariot® complexes were prepared with 8 μL of 1 mg/mL of protein and 20 μL of Chariot® and added to the DRGs for 1 hr at 37° C as described above for NG108-15 cells. At the end of the hour, another 500 μL of Neurobasal medium was added. The cultures were imaged after 48 hour as described below and then fixed with 4% paraformaldehyde.

3.2.4. Quantification of Neurite Crossing

The NG108-15 cells and DRGs were imaged and analyzed using ImagePro software (Media Cybernetics, Carlsbad, CA) through a Magnafire CCD camera (Optronics, Goleta, CA) attached to an inverted Nikon T300 microscope. For the cultures with NG108-15 cells, the neurites were counted and categorized into two groups: (1) neurites that extended along LN lanes and (2) processes that crossed into the CSPG lanes. Neurites were counted only in the regions where cells attached and formed distinct lanes. A percent ratio of neurites crossing the CSPG lane was calculated based on the equation: $\frac{\#\text{ of neurites crossing into CSPG lane}}{\#\text{ of neurites growing on the LN lanes}} * 100$. For the cultures with whole DRGs, the results were quantified in a blinded state by two, independent, unbiased people. Each DRG explant in the cultures was scored from 0 to 4, similar to the scoring system used by

Walter et al to quantify the amount of neurite crossing from retinal explants (Walter et al. 1987). The score of 0 is given to explants that exhibit a preference for either LN or CSPG substrate and 4 is given to explants with neurites that show no preference for either of the two substrates. The scores 1-3 were given for intermediate neurite crossing. At least 3 DRGs per condition were analyzed by each of the two blinded people and their scores were averaged.

3.2.5. Statistical Analysis

The Student t-test was performed using Minitab software. Conditions were considered to be statistically different for p-values < 0.05.

3.3. RESULTS

3.3.1. Percent Ratio of Neurites Crossing after Transduction of Rho GTPases

The percent ratio of neurite crossing into the CSPG lanes was measured for the two negative controls (no protein transduction and GST transduction), as well as Chariot® complexed to C3 and the Cdc42, Rac1, and RhoA mutants (Fig.3.1). The two negative controls, either containing only media or GST protein, complexed with the Chariot®, displayed few neurites crossing the CSPG lanes (11.3% and 8.9%, respectively). The mutant protein, CA-Rac1, had the highest percentage of crossing at 41.1%, followed by CA-Cdc42 and C3, which had crossing percentages of 31.3% and 28.1%, respectively, which were all statistically significant compared to the negative controls.

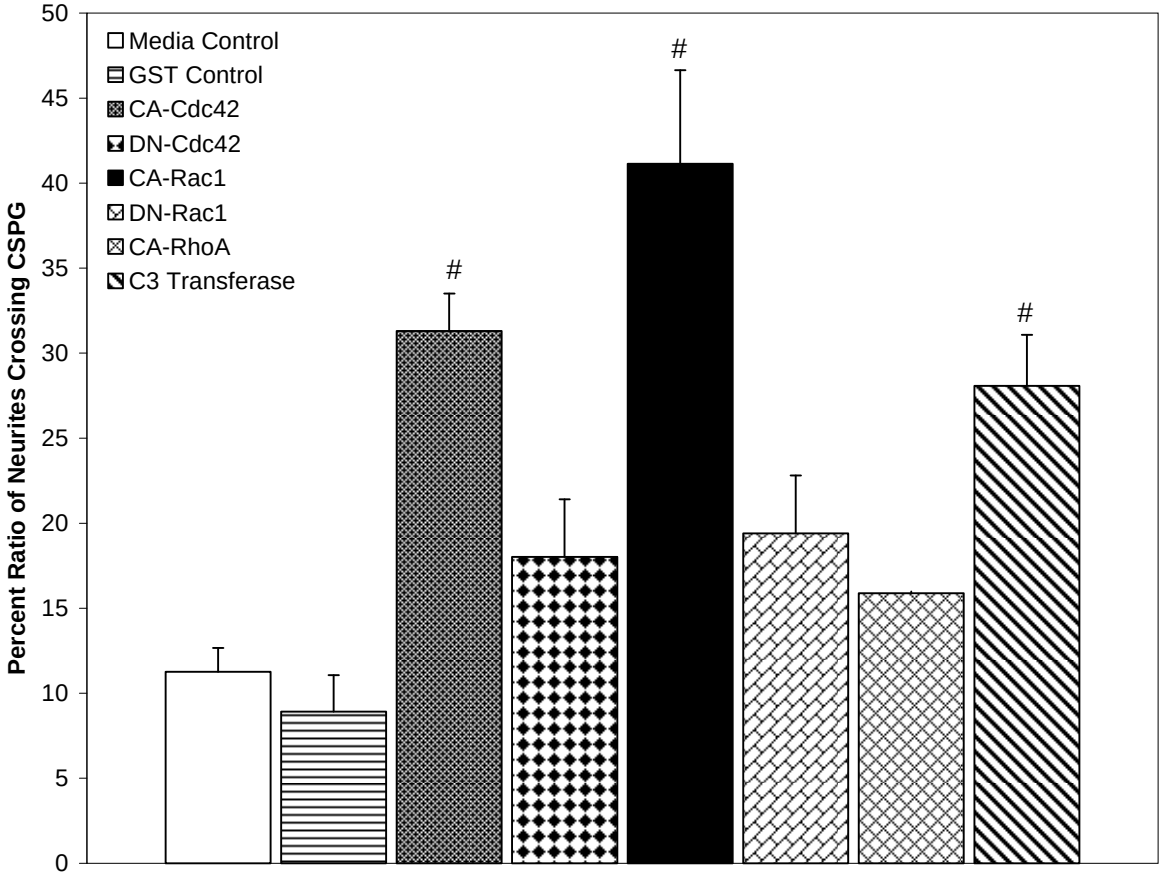


Figure 3.1 Percent ratio of neurites crossing for the controls, Rho GTPase mutant proteins, and C3 transferase. After treatment with CA-Cdc42, CA-Rac1, or C3, the percent ratio of neurites crossing CSPG increased. The data represent the mean + S.E.M. for three experiments. # Statistically different compared to media control (p-value < 0.05).

In Figure 3.2, phase micrographs illustrate the effect of the mutant proteins, C3, and the negative controls. The neurites extended on the LN lane or along the CSPG/LN border under the control conditions (Fig. 3.2A and B). CA-Cdc42, CA-Rac1, and C3 had the greatest amount of neurites crossing the CSPG lane (Fig. 3.2C, D, and E). The dominant negative mutants and CA-RhoA had little effect on neurite extension (Fig. 3.2F, G, and H).

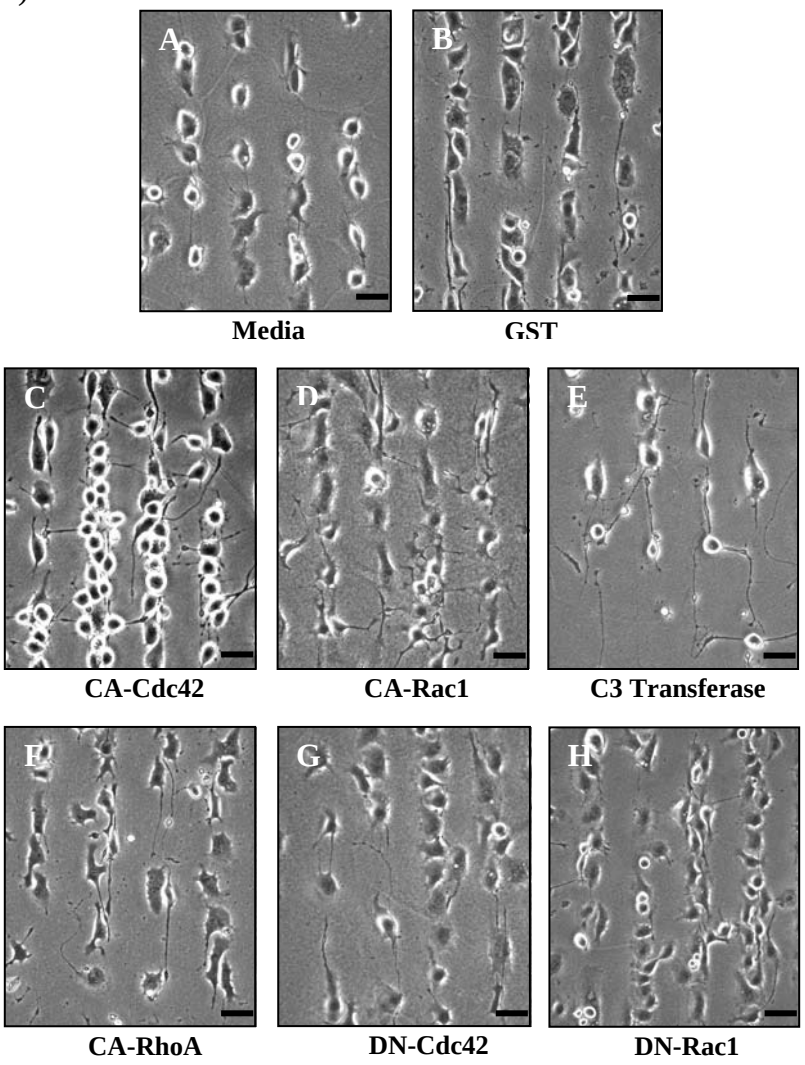


Figure 3.2 NG108-15 cells transduced with mutant Rho GTPases and C3. (A) Media control, (B) GST control, (C) CA-Cdc42, (D) CA-Rac1, (E) C3 transferase, (F) CA-RhoA, (G) DN-Cdc42, and (H) DN-Rac1. The neurons attached on the laminin lanes and the neurites predominantly grew along the laminin/CSPG border. CA-Cdc42, CA-Rac1, and C3 (C-E) induced neurites to cross the CSPG lanes. The neurites in the two controls, DN-Cdc42, DN-Rac1, and CA-RhoA (A,B, F-H) sample groups grew on the laminin lanes with very little to no crossing of the CSPG lanes. Scale Bars = 50 μm.

3.3.2. Optimal Dosage for Percent Ratio of Neurite Crossing

A dose response experiment was conducted on CA-Cdc42 using 6 different protein concentrations; 1 μg/mL, 2 μg/mL, 3 μg/mL, 4 μg/mL, 6 μg/mL, and 8 μg/mL (Fig. 3.3). Only the lower four concentrations are plotted on this graph as the higher doses (6 μg/mL, and 8 μg/mL) were toxic and did not permit cell attachment. As shown in Fig. 3, the percent ratio of crossing was 14.7%, 14.4%, and 12.7% for 1 μg/ mL, 2 μg/ mL, and 3 μg/mL CA-Cdc42 sample groups, respectively. The concentration that elicited the highest percent ratio of crossing was 4 μg/mL CA-Cdc42 (31.3%). Only the 4 μg/mL CA-Cdc42 sample group was statistically different from the media control.

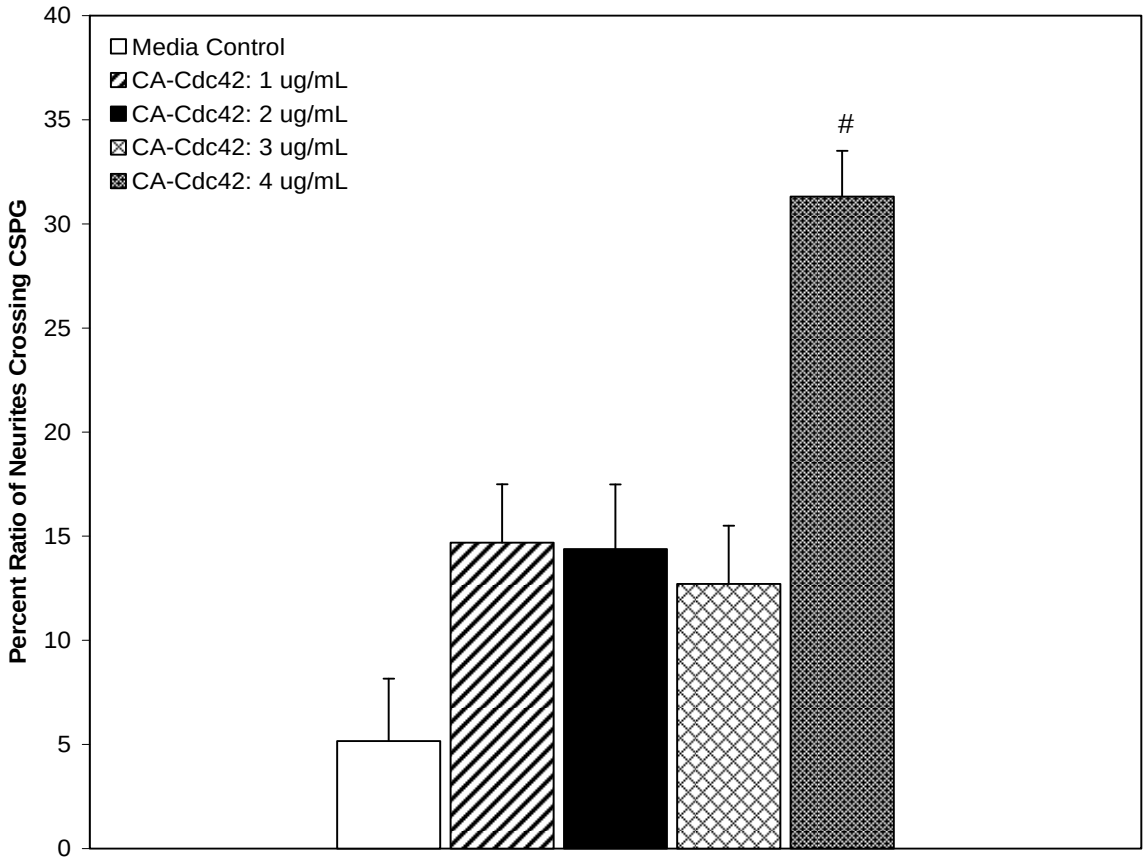


Figure 3.3 CA-Cdc42 dose response curve for NG108-15 cells. CA-Cdc42: 4 μg/mL induced the highest percent ratio of neurites crossing the CSPG lanes. The data represent the mean + S.E.M. for three experiments. # Statistically different compared to media control (p-value < 0.05).

3.3.3. Effect of Chariot® Transduction System

Figure 3.4 graphically portrays the effect of using the Chariot® transduction system. CA-Rac1 was used to investigate whether Chariot® was necessary to aid protein uptake by the cells. Figure 3.4 demonstrates that there was a significantly greater number of neurites that crossed the CSPG lane when the mutant protein was complexed to the delivery reagent compared to the negative control. The average percent ratio of crossed neurites through the inhibitory region in the absence of Chariot® was lower than the media control with an average of 6.9%.

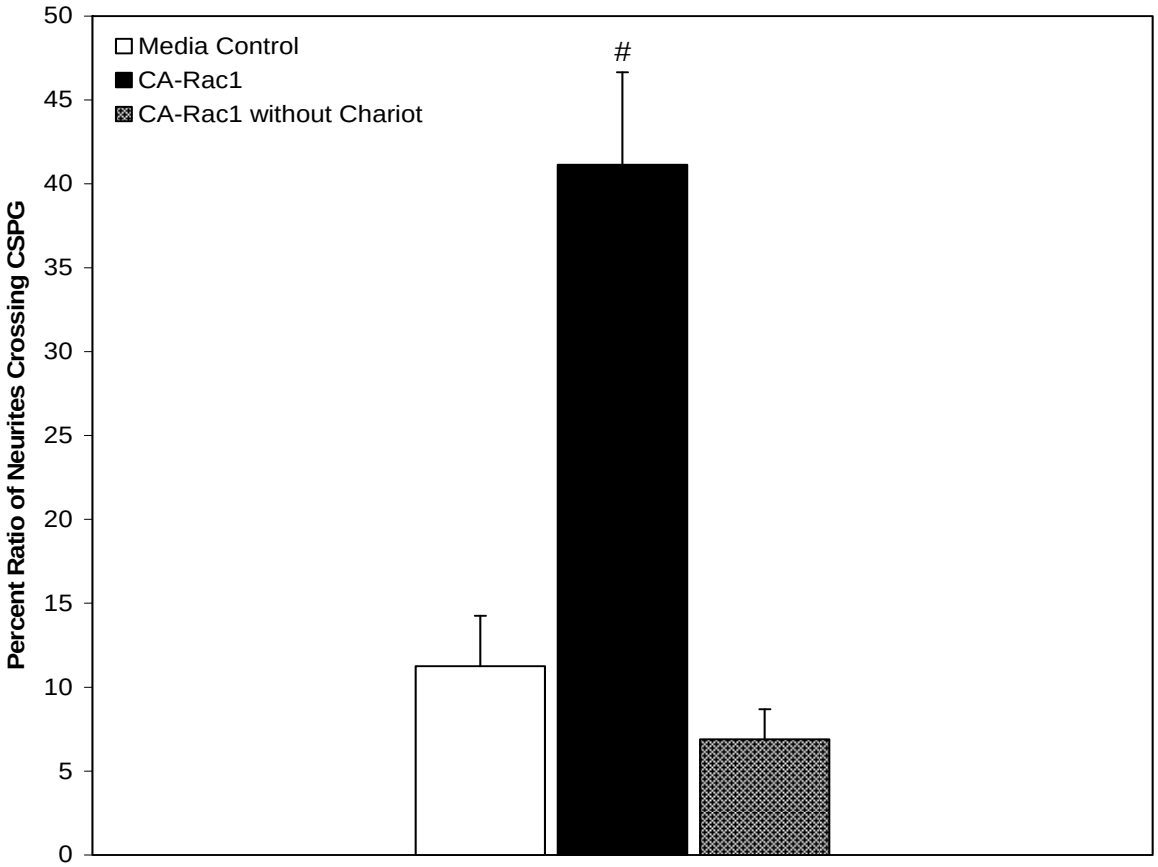


Figure 3.4 Percent ratio of neurites crossing after CA-Rac1 treatment with and without Chariot®. CA-Rac1 with Chariot® elicited the highest percentage of crossing. CA-Rac1 without the Chariot® had a similar percent ratio as the media control. The data represent the mean + S.E.M. for three experiments. # Statistically different compared to media control (p-value < 0.05).

C3 was administered as the molar equivalent of the other mutant proteins (Fig. 3.1). However, in a separate experiment, the molar amount of C3 was doubled (4 μg/mL) to observe the effect on neurite extension through the CSPG lanes. When comparing the different C3 sample groups to the media control, 2 μg/mL and 4 μg/mL of C3 were statistically greater (Fig. 3.5). C3 was added to the cells without Chariot as well. The percent ratio of neurites crossing was similar to the media control (13%).

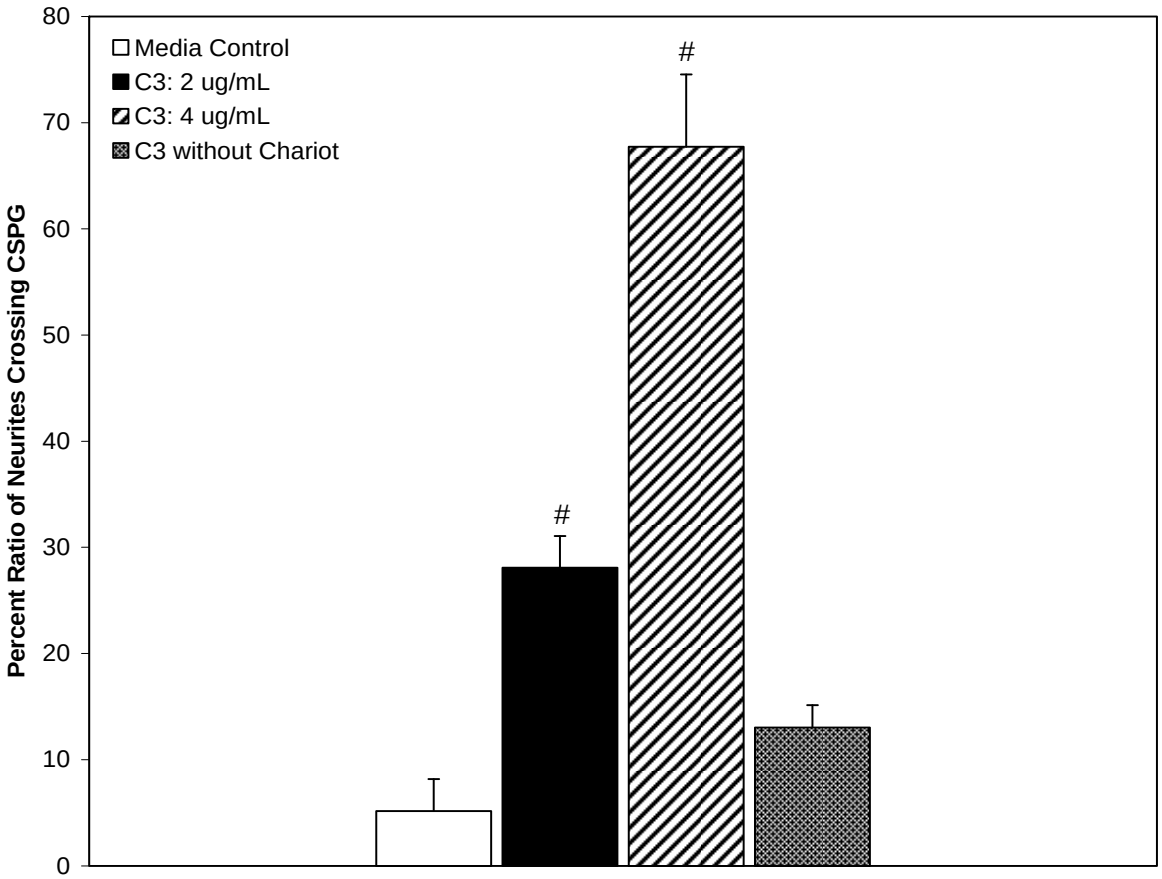


Figure 3.5 C3 transferase with and without Chariot®. When the dosage of C3 was doubled, the percent ratio of crossing increased. Without the Chariot® the percent ratio of neurites crossing is similar to the media control. The data represent the mean + S.E.M. for three to four experiments. # Statistically different compared to media control (p-value < 0.05).

3.3.4. Transduction of Rho GTPases into DRGs

To determine whether or not the effect observed in the NG108-15 cells would be similar in primary neurons, CA-Cdc42, CA-Rac1, DN-Rac1, and C3 proteins were transduced into whole E9 chick DRGs. Figure 3.6 depicts the results for whole DRGs neurites extending along the LN and CSPG regions. A modified version of the preference scoring system developed by Walter et al (Walter et al. 1987) was used for analysis. The results demonstrate that in the media control, the neurites had a score of 1.2 with a strong preference for the laminin regions. CA-Cdc42 and CA-Rac1 had a score of 3 and 2.9, respectively, with greater number of neurites crossing over into the CSPG regions. The neurite crossing for C3 scored a 2 and DN-Rac1 scored a 1.5. In Figure 3.7, phase micrographs of the DRG cultures at 48 hours after plating are shown. The lower portion of each image distinguishes between LN lanes (grey) and CSPG lanes (black). The media control shows the majority of the neurites in tight bundles growing along the LN regions with a few neurites crossing the CSPG lanes (Fig. 3.7A). Bundles of neurites extending along the LN lanes can also be seen in the sample groups when CA-Cdc42, C3, or CA-Rac1 was transduced into the E9 DRGs, however, there are also increased amounts of neurites that are crossing the CSPG inhibitory lanes (Fig. 3.7B, C, and D). The sample group that was transduced with DN-Rac1 was similar to the media control, with neurites growing along the LN lanes and very few crossing into the CSPG lane (Fig. 3.7E). Therefore, CA-Cdc42, C3, and CA-Rac1 increased the number of neurites extending into the CSPG lane in both NG108-15 cells and primary DRGs.

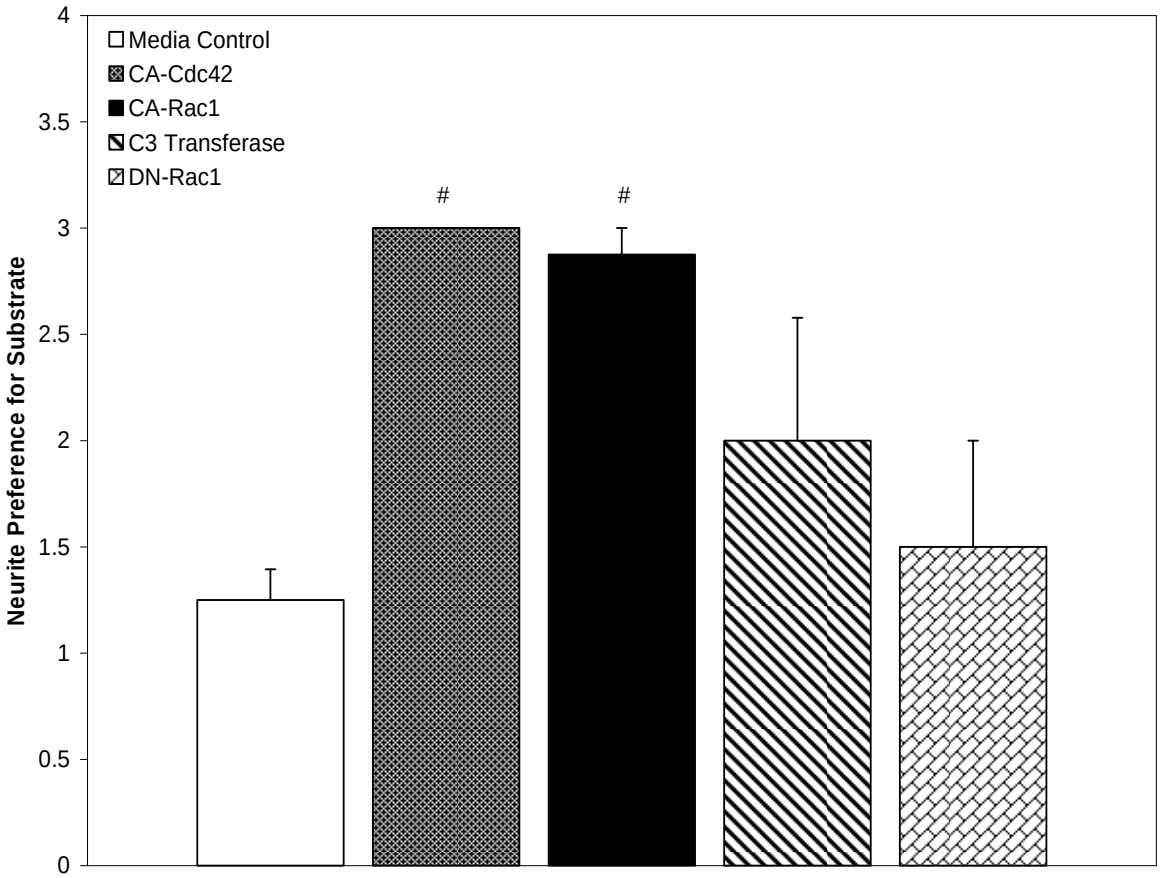


Figure 3.6 DRG neurite preference for a substrate. A score of 0 means that neurites preferred to extend on only one substrate, either LN substrate or CSPG. A score of 4 means that the neurites do not show a preference for a specific substrate. A score between 1 and 3 indicates that there is a preference for one substrate, however, the neurites will extend along the other regions. After treatment with CA-Cdc42 and CA-Rac1, the neurites show less of a preference for laminin and extended along the CSPG lanes. The data represent the mean + S.E.M. for three experiments. # Statistically different compared to the media control (p-value < 0.05).

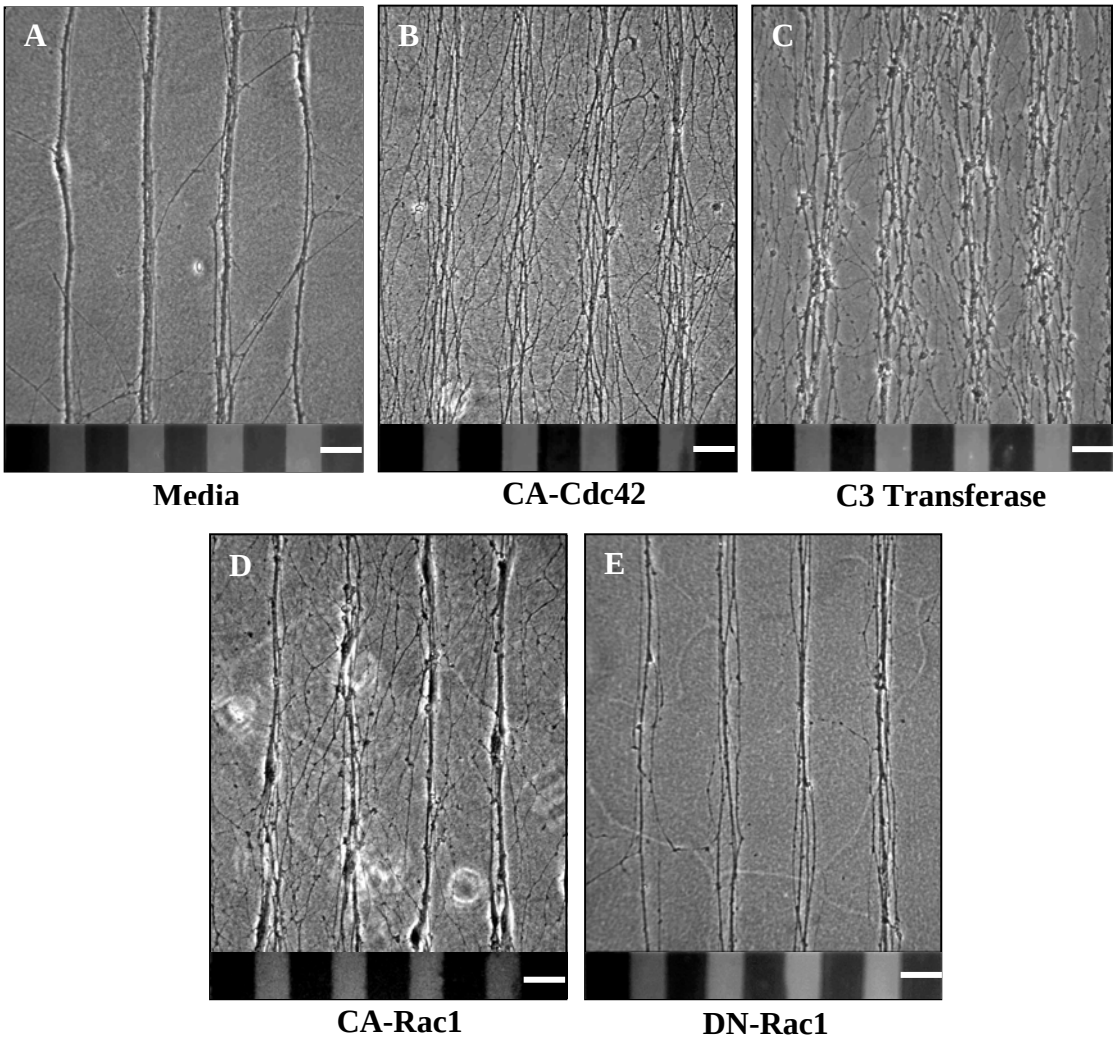


Figure 3.7 DRGs transduced with mutant Rho GTPases and C3. (A) Media control, (B) CA-Cdc42, (C) C3 transferase, (D) CA-Rac1, and (E) DN-Rac1. Three to five experiments were conducted per condition. Note that under each neurite image is an image that shows LN lanes in gray and CSPG lanes ιν βλαχκ. Σιμιλαρ το τηε ΝΓ10815 χελλσ, ΧΑ–Χδχ42, ΧΑ–Ραχ1, ανδ Χ 3 (Β–Δ) ινδυχεδ νευριτεσ το χροσσ τηε ΧΣΠΓ λανεσ. Τηε νευριτεσ ιν τηε με δια χοντρολ ανδ τηε ΔΝ–Ραχ1 (Α ανδ Ε) σαμπλε γρουπσ γρεω ον τηε λαμινι ν λανεσ ωιτη ωερψ λιττλε χροσσινγ. Σχαλε Βαρσ = 50 μm.

3.4. DISCUSSION

Nerve regeneration in the CNS is currently limited. Our hypothesis is that CSPGs in glial scars are partially responsible for CNS regenerative failure by specifically sending inhibitory signals to the neurons, blocking regeneration. Understanding the signaling pathways involved in blocking neurite outgrowth through the scar has been difficult. However, knowledge of the signals involved in this region would illuminate the strategies that promote regeneration through the scar. We hypothesized that regardless of the precise nature of the signals, they must converge on molecules that regulate the actin cytoskeleton, which are necessary for neurite outgrowth. The Rho GTPases represent a point of convergence for many extracellular signals that regulate the actin polymerization. Our strategy was to either block the inhibitory signals or mimic positive signals to stimulate neurite extension through inhibitory interfaces. Therefore, we modulated the activity of the Rho GTPases (Rac1, Rho and Cdc42), which play important roles in regulating the actin cytoskeleton (Hall and Nobes 2000); (Luo 2000). The Rho GTPases have also been implicated in axon guidance and in neurite outgrowth (Kuhn et al. 2000). We demonstrate that this strategy is effective in overcoming CSPG-dependent inhibition of neurite outgrowth in an *in vitro* model system. We demonstrate that activation of Rac1 and Cdc42, as well as inhibition of Rho, promotes neurite outgrowth through CSPG regions. We speculate that the CSPG-mediated inhibition was alleviated either by modulating intracellular signaling to frustrate CSPG's inhibitory role, or by altering the cytoskeletal mechanics so that repulsive forces was overcome.

Specifically, the effect of the mutant Rho GTPases proteins on the NG108-15 neurites' ability to cross into CSPG regions was quantified (Fig. 3.1). All the mutant

proteins were transduced into the NG108-15 cells using the Chariot® system. The proteins that promoted neurite crossing, C3, CA-Cdc42, and CA-Rac1, had significantly higher percent ratio of neurite crossing into the CSPG lanes compared to the negative controls. CA-Cdc42 and CA-Rac1 were also statistically greater than DN-Cdc42, DN-Rac1, and CA-Rho, indicating that these two proteins in their active state promote neurite extension into CSPG regions. Through the inactivation of RhoA signal transduction pathway, C3 had a significantly higher number of neurites crossing than DN Cdc42 and CA-RhoA when 2 μg/mL of C3 was transduced. When a higher concentration of C3 (4 μg/mL) was transduced, the condition was statistically greater than DN-Cdc42, DN-Rac1, and CA-RhoA. Therefore, these results demonstrate that transducing CA-Rac1 and CA-Cdc42 complexed to Chariot®, as well as C3, can increase the number of neurites extending from the LN lanes into the CSPG lanes.

To determine if combinations of mutant proteins produced synergistic effects, CA-Cdc42 and CA-Rac1 were cotransduced into the same lane modified culture dish such that the total mutant protein concentration was the same as with the single protein alone (4 μg/mL) combining 2 μg/mL of CA-Cdc42 with 2 μg/mL of CA-Rac1. The percent ratio of crossing for this condition was around 13% (data not shown). This percent ratio is similar to that observed when 2 μg/mL is transduced alone (Fig. 3.3). When higher concentrations of CA-Cdc42 and CA-Rac1 were cotransduced into the NG108-15 cells (total concentrations of 6 μg/mL and 8 μg/mL), the cells did not survive, although they remained attached to the surface, probably due to toxicity associated with the protein transduction. Therefore, there was no observed synergistic effect of adding CA-Cdc42 and CA-Rac1.

To determine if there was a dose response to the mutant protein, six different concentrations of CA-Cdc42 were added (Fig. 3.3). CA-Cdc42 was chosen for the dose response curve due to its positive effect on neurites crossing the CSPG lanes (Fig. 3.1). At the higher concentrations (6 μg/mL and 8 μg/mL), cell death occurred probably due to cytotoxicity either due to the protein itself or the Chariot® reagent. A higher concentration, basic proteins in the cell such as the Rho GTPases, may potentially trigger a signal to begin apoptosis, leading to cell death. The lower three concentrations of protein did not evoke a substantial increase compared to the negative controls in the percent average of the neurites crossing the lanes. The narrow range of protein concentration that leads to cellular responses, shows that there is an optimal concentration to observe the desired response. Therefore, the percentage ratio of neurites crossing the CSPG lane was more of a rectangular response rather than a peak response. The percent of neurites crossing in wells that were transduced with the lower three concentrations of CA-Cdc42 were statistically lower when compared to dishes with 4 μg/mL of CA-Cdc42, which produced an average neurite crossing that was 2-fold higher. Therefore, the concentration of 4 μg/mL of CA-Cdc42 was optimal to elicit a crossing response from the neurons in our culture conditions. This validates the mutant protein concentrations used for the experiment comparing the effects of all of the Rho GTPase mutants.

Three commonly utilized methods to express a specific protein in cells, trituration (Jin and Strittmatter 1997), microinjection (Paterson et al. 1990), and scrape loading (Lehmann et al. 1999), are aggressive and can disrupt normal cell function. Therefore, it is necessary to engineer a method of transducing proteins efficiently without risking cell

viability and administering the proteins *in vivo*. In some studies, the TAT peptide was used to aid in protein transduction (Winton et al. 2002); (Dubreuil et al. 2003); (Monnier et al. 2003). In this study, the Chariot® reagent was used to transduce the proteins across the cellular membrane. Thus far, none of the previous studies have complexed protein with a generic delivery vehicle to aid in the protein transduction. To investigate the contribution of Chariot®, both CA-Rac1, and C3 (two of the three conditions with the highest percent crossing through the CSPG lanes (Fig. 3.4) were added to the neurons without any Chariot® (Fig. 3.4 and 3.5). Figure 3.4 shows that CA-Rac1 complexed with Chariot® had a 6-fold higher percent ratio of neurites crossing the lane compared to when the protein was added directly to the cultures, which makes it significantly higher. When the Chariot® was excluded during the addition of C3, the percent ratio of crossing decreased by more than a 2-fold (Fig. 3.5). These experiments confirm that the Rho GTPase mutant proteins as well as C3 have a limited ability to enter the cells on their own and need the aid of a delivery vehicle to enter the cells and influence neurite extension on CSPG.

C3 was the only protein transduced into the neurons that is not a member of the Rho GTPase family. The other mutants are constitutively active or dominant negative forms of the Rho GTPases. However, C3 is used to inhibit the activation of the RhoA. The molecular weight of C3 (24 kDa) is approximately half that of the other mutant proteins (50-52 kDa), therefore, 2 μg/mL was used in a previous experiment as a molar equivalent (Fig.3.1). To observe the effect of doubling the molar concentration, 4 μg/mL of C3 was complexed to Chariot®. The percent ratio increased by more than a 2-fold (Fig. 3.5). The percent ratio of neurite crossing after transducing 4 μg/mL of C3 was

significantly greater than 2 μg/mL of C3 and both of these conditions were statistically greater than C3 without the Chariot® reagent. This demonstrates that a higher concentration of C3 can promote greater number of neurites crossing the inhibitory CSPG lanes *in vitro*.

Most studies investigating Rho GTPases have focused on RhoA, rather than examining Rac1 and Cdc42-dependent regulation of regeneration. The addition of C3 or Y27632 blocks myelin and myelin/CSPG inhibition *in vitro* (Dergham et al. 2002), as well as CSPG inhibition (Borisoff et al. 2003). Another study demonstrated that on NOGO and myelin substrates, the percentage of neurite outgrowth increased after the administration of C3 (Fournier et al. 2003). In *in vitro* stripe assay experiments, C3 was fused to TAT and the studies demonstrated that inactivating the Rho pathway overcame CSPG inhibition (Monnier et al. 2003), as well as myelin (Winton et al. 2002) and NOGO (Dubreuil et al. 2003). The use of TAT as a delivery vehicle requires a tedious process to construct the mutant protein. Chariot® is convenient to use because it is a generic delivery reagent that can be complexed to any protein and works with high efficiency. There has been conflicting evidence on the benefits of adding C3 into the corticospinal tract in order to achieve long length regeneration (Dergham et al. 2002; Fournier et al. 2003). One of the theories for C3 failing to generate axon growth over long-distance gaps is that the Rho inhibitor is not successfully transduced into the injured cells at the site (Fournier et al. 2003).

E9 whole DRGs were cultured to determine whether the results obtained with the NG108-15 cells could be replicated in primary neurons. CA-Cdc42 and CA-Rac1 were transduced into the cells and induced a significantly greater amount of crossing similar to

the NG108-15 cells. This demonstrates that although neurites preferred the LN substrate, after CA-Cdc42 or CA-Rac1 was transduced, the neurites were manipulated to extend on CSPG lanes Although C3 was not significantly different compared to the media control, transducing the protein did evoke a neurite crossing into the CSPG region. DN-Rac1 was used as the negative control in this experiment and did not encourage neurites to cross into the inhibitory region, which is shown in Figures 3.6 and 3.7. Obtaining similar results from two different cell types allows the conclusion that CA-Cdc42, CA-Rac1, as well as C3 stimulate growth cone extension into the CSPG lane and overcome its inhibitory effects. It would be interesting to evaluate the utility of CA-Cdc42 and CA-Rac1 transduction *in vivo* to overcome regenerative failure in the CNS.

3.5. ACKNOWLEDGMENTS

We would like to thank Sonya Ensslen and Paras Parikh for their contribution in the production of the modified surfaces and Wei He and Rupal Thazhath for their help with the blinded analysis with DRG experiments.

3.6. REFERENCES

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W. 2003. Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci 22(3):405-416.

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(6881):636-640.

Chen ZJ, Ughrin Y, Levine JM. 2002. Inhibition of axon growth by oligodendrocyte precursor cells. Mol Cell Neurosci 20(1):125-139.

Condic ML, Lemons ML. 2002. Extracellular matrix in spinal cord regeneration: getting beyond attraction and inhibition. Neuroreport 13(3):A37-48.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81(7):1137-1146.

David S, Lacroix S. 2003. Molecular Approaches to Spinal Cord Repair. Annu Rev Neurosci.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. 2002. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22(15):6570-6577.

Dubreuil CI, Winton MJ, McKerracher L. 2003. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. J Cell Biol 162(2):233-243.

Fawcett JW, Asher RA. 1999. The glial scar and central nervous system repair. Brain Res Bull 49(6):377-391.

Filbin MT. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4(9):703-713.

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23(4):1416-1423.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509-514.

Hall A, Nobes CD. 2000. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci 355(1399):965-970.

Hoke A, Silver J. 1996. Proteoglycans and other repulsive molecules in glial boundaries during development and regeneration of the nervous system. Prog Brain Res 108:149-163.

Jin Z, Strittmatter SM. 1997. Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17(16):6256-6263.

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(6):1566-1569.

Kozma R, Ahmed S, Best A, Lim L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15(4):1942-1952.

Kuhn TB, Meberg PJ, Brown MD, Bernstein BW, Minamide LS, Jensen JR, Okada K, Soda EA, Bamburg JR. 2000. Regulating actin dynamics in neuronal growth cones by ADF/cofilin and rho family GTPases. J Neurobiol 44(2):126-144.

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L. 1999. Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19(17):7537-7547.

Luo L. 2000. Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci 1(3):173-180.

McKeon RJ, Hoke A, Silver J. 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136(1):32-43.

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(4):805-811.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22(3):319-330.

Morgenstern DA, Asher RA, Fawcett JW. 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 137:313-332.

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(3):757-767.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE. 2002. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22(23):10368-10376.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A. 1990. Microinjection of recombinant p21rho induces rapid changes in cell morphology. J Cell Biol 111(3):1001-1007.

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):1-11.

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.

Snow DM, Smith JD, Gurwell JA. 2002. Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. J Neurobiol 51(4):285-301.

Spencer T, Domeniconi M, Cao Z, Filbin MT. 2003. New roles for old proteins in adult CNS axonal regeneration. Curr Opin Neurobiol 13(1):133-139.

Tang BL. 2003. Inhibitors of neuronal regeneration: mediators and signaling mechanisms. Neurochem Int 42(3):189-203.

Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA. 1990. In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp Brain Res 81(2):283-287.

Walter J, Kern-Veits B, Huf J, Stolze B, Bonhoeffer F. 1987. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. Development 101(4):685-696.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L. 2002. Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277(36):32820-32829.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D. 1998. Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Exp Neurol 154(2):654-662.

CHAPTER 4

DELIVERY AND CHARACTERIZATION OF BDNF, CA-CDC42, AND CA-RAC1 TO PROMOTE AXONAL OUTGROWTH THROUGH CSPG-INHIBITORY REGIONS AFTER SPINAL CORD INJURY

(Partially as published with Y.T. Kim, R.J. McKeon, and R.V. Bellamkonda, Biomaterials, 27(2006) 497-504 and manuscript in preparation with S.M. Brady-Kalnay, R.J. McKeon, and R.V. Bellamkonda)

Spinal cord injury often results in permanent functional loss. After injury, a series of events occur around the lesion site, including the deposition of growth cone inhibitory astroglial scar tissue containing chondroitin sulfate proteoglycan (CSPG)- rich regions. It is important to encourage axons to extend through these inhibitory regions for regeneration to occur. In this study, the Rho GTPases, Cdc42 and Rac1, which are involved in actin cytoskeleton dynamics, were locally delivered in constitutively active (CA) form into a hemisectioned lesion site. Additionally, BDNF was delivered to the lesion site, either individually or in combination with either CA-Cdc42 or CA-Rac1. The dorsal over-hemisection model was utilized, creating a ~2mm defect that was filled with an *in situ* gelling hydrogel scaffold containing lipid microtubules loaded with the protein(s) to encourage axonal outgrowth. The lipid microtubules enable slow release of proteins while the hydrogel serves to localize them to the lesion site and permit axonal outgrowth. The results from this study demonstrate that groups treated with BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 had significantly higher

percentage of axons from the corticospinal tract (CST) that traversed the CSPG-inhibitory regions proximal to the lesion site, as well as penetrate the glial scar compared to the untreated and agarose controls. Although axons from the CST tract did not infiltrate the scaffold-filled lesion, NF-160-positive axons were observed in the scaffold. Treatment with BDNF, CA-Cdc42, and CA-Rac1 also reduced the reactive astrocytes and CSPG deposition, quantified by analyzing GFAP and CS-56 intensity, respectively, at the interface of the scaffold and host tissue. Therefore, the local delivery of CA-Cdc42, CA-Rac1 and BDNF, individual and combination demonstrated the ability of axons to extend through CSPG inhibitory regions proximal to the lesion site, as well as reduce the glial scar components.

4.1. INTRODUCTION

Injuries to the CNS trigger a cascade of events, typically resulting in permanent functional loss. In order for axonal regeneration to occur in the CNS after injury, a microenvironment suitable for regeneration is critical (Evans 2001). One strategy is to design a suitable biomaterial scaffold that can be implanted at the site of injury. In this study we report the design and evaluation of an agarose based scaffold with two-elements: (1) a three-dimensional (3D) scaffold that is optimized to allow axonal growth in 3D, and (2) an embedded drug delivery system for sustained, local release of neurotrophic factors. Although not included in this study, these scaffolds also have the potential to present growth promoting extracellular matrix (ECM) proteins such as laminin-1 to the lesion site.

Due to their soft, tissue-like mechanical properties, agarose, alginate and collagen hydrogels have been investigated as potential scaffolds in the CNS and in the peripheral nervous system(Dubey et al. 1999; Houweling et al. 1998; Kataoka et al. 2004; Labrador et al. 1998). Specifically, our laboratory has utilized agarose as a scaffold for neural tissue engineering for a few reasons: 1) it is biocompatible and does not cause an adverse reaction when implanted *in vivo*; 2) by manipulating the porosity and mechanical properties, it can be optimized for maximum axonal outgrowth (Balgude et al. 2001; Bellamkonda et al. 1995b; Dillon et al. 2000); 3) it can support cell migration; and (4) it can be utilized as part of a trophic factor delivery system with embedded sustained release vehicles (Meilander et al. 2001), as well as be used to bind protein to its backbone for spatial control (Bellamkonda et al. 1995a; Borkenhagen et al. 1998; Yu and Bellamkonda 2003). However, *in situ* gelling agarose hydrogels have previously not been extensively used in the CNS, specifically to bridge spinal cord defects before, and in this study we report the response of spinal CNS tissue to implanted agarose hydrogels and to local delivery of the Rho GTPases, Cdc42 and Rac1, and BDNF.

Intrinsic and extrinsic strategies can be used to overcome the inhibitory astroglial environment to promote axonal outgrowth. Intrinsic strategies involve modulating proteins or genetic material into neurons to promote axonal outgrowth, where as extrinsic methods remove the inhibitory components from the microenvironment. Intrinsic therapies have consisted of transducing Rho GTPases and cAMP. Rho GTPases, Cdc42 and Rac1, are responsible for the filopodial and lamellopodial extension on the growth cone (Hall 1998; Nobes and Hall 1995), where as activation of Rho leads to growth cone collapase (Hall 1998). It has previously been demonstrated by our laboratory and other

groups that when CA-Cdc42 and CA-Rac1 or molecules that inhibit Rho and its signaling pathway were transduced into neurons *in vitro*, neurites extended through inhibitory regions (Jain et al. 2004; Jin and Strittmatter 1997; Monnier et al. 2003; Winton et al. 2002). Studies were performed where inhibitors to Rho and ROCK were delivered *in vivo*, preventing growth cone collapse, thus promoting axonal regeneration (Dergham et al. 2002). However, in a similar study, significant axonal regrowth was not observed after C3 transferase transduction (Fournier et al. 2003). In this study, CA-Cdc42 and CA-Rac1 are locally delivered into the lesion site after SCI. Studies that have delivered Cdc42 or Rac1 after SCI have not been published.

Neurotrophic factors play a significant role in axonal regeneration, as well as in neural development, survival and outgrowth. Neurotrophic factors include BDNF, NGF, NT-3 (Schmidt and Leach 2003). After SCI, retrograde degradation occurs, which induces neuronal death in the tracts that have been transected. Specifically the three neurotrophic factors listed above, BDNF, NGF and NT-3, exhibit neuroprotective behavior due to their influence on motor and sensory neuronal survival (Novikova et al. 2000), as well as on regenerative outgrowth of sensory fibers (Oudega and Hagg 1999; Zhou and Shine 2003). It has been demonstrated that delivery of neurotrophins several weeks post-injury resulted in improvement in neuronal survival (Novikova et al. 2000), and aided in regeneration of fibers in the spinal cord. Sustained local delivery may be important to generate these beneficial effects as it has been shown that a single injection of trophic factors into the brain has a limited half-life of 30 minutes (Krewson et al. 1995). Also, in the case of NT-3, it was demonstrated that continuous infusion rather than a single injection provided a neuroprotective component (Sayer et al. 2002).

Therefore, slow release of neurotrophins should be an integral component of any strategy to achieve CNS axonal regeneration to ensure their activity over the prolonged period of time (weeks to months) that is likely necessary for fibers to regenerate. Technology for sustained local release of proteins, specifically neurotrophic factors, has primarily been focused on the use of osmotic pumps. The disadvantage of using the osmotic pump is that they are capable of producing additional damage at the site of implantation and require having their protein reservoir replaced. To address some of these shortcomings, our laboratory has reported the use of lipid microtubular slow release vehicles that can be embedded in hydrogels to achieve sustained release. We have previously reported that lipid microtubules can deliver DNA and proteins *in vitro* and *in vivo* (Meilander et al. 2003; Meilander et al. 2001; Yu and Bellamkonda 2003). The microtubules are composed of multiple planar lipid bilayers that form hollow cylinders when they are loaded with a solution (Schnur 1993; Shimizu 2002). The microtubules are 40 μm in length and 0.5μm in diameter and release the protein from the ends of the tubule. Several parameters including the initial loading concentration, the length of the microtubules, and the mass of microtubules/ml can be modulated to achieve a desired release profile.

In this study, the hydrogel scaffold and embedded lipid microtubule delivery system was used to deliver the CA-Cdc42 and CA-Rac1, and BDNF individually and in combination. We investigated the *in situ* gelling capabilities of an agarose hydrogel to determine whether it would conform to the shape of the spinal cord cavity without inducing an inflammatory response besides what occurs due to injury. This study demonstrates that the agarose conforms to the shape of the spinal cord cavity as it gels *in*

situ, 37°C, and provides a scaffold for BDNF delivery without inducing an inflammatory response. Our results demonstrated that the Rho GTPases and BDNF increased the percentage of axons that traversed the inhibitory regions proximal to the glial scar compared to the untreated and agarose controls. Also, CA-Cdc42 reduced the inflammatory response, reactive astrocytes and CSPG deposition, while CA-Rac1, BDNF, BDNF/CA-Rac1, and BDNF/CA-Cdc42 decreased axonal retraction from the lesion site.

4.2. MATERIALS AND METHODS

4.2.1. Agarose Gelling Temperature

SeaKem®, SeaPlaque®, and SeaPrep® agarose (Cambrex Bio Science Rockland Inc., Rockland, ME) samples of 0.3%, 0.5%, and 1% (wt/vol) were prepared using methods similar to those previously reported (Dillon et al. 1998) Briefly, for example, 100 mg of SeaKem® agarose was dissolved in 10 ml of 300 mM PBS at 90 °C. Two hundred microliters of agarose solution was loaded onto the stage of the Bohlin CVD Rheometer (Bohlin, East Brunswick, NJ). Upon cooling, a parallel plate configuration was utilized in the Rheometer with the sample being subjected to a constant frequency of 0.05 Hz and low amplitude shear stress of 1.5 Pa. A temperature sweep was performed starting at 50 °C and decreased to 0 °C to quantify the specific temperature at which the agarose solution becomes a gel.

4.2.2. Neurite Extension of DRGs in 3D Agarose Gels

Whole embryonic day 9 (E9) White Leghorn Chick dorsal root ganglia (DRG) were cultured in various concentrations of agarose. The agarose was prepared as mentioned above. To develop the 3D scaffolds, 200 μL of agarose was initially added to a 24 well and gelled at 4 °C for 20 min. The DRGs were then mixed with 200 μL of agarose and added above the initial agarose layer. One milliliter of Neurobasal media (Invitrogen, Carlsbad, CA), supplemented with B-27, L-glutamine, and 1% penicillin streptomycin, along with 1 μg/mL of NGF was added to each culture well.

4.2.3. Fabrication and Loading of Lipid Microtubules for Sustained Release of CA-Cdc42, CA-Rac1, and BDNF

The methodology to fabricate the lipid microtubules was previously published (Meilander et al. 2003; Meilander et al. 2001). Briefly, 1,2-bis-(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$, Avanti Polar Lipids, Alabaster, AL) was dissolved in 70% ethanol at a concentration of 1 mg/mL. The lipid was placed in a water bath (Thermo NESLAB, Portsmouth, NH) programmed to decrease from 50 to 20°C over 48 h and then stored at room temperature to facilitate self-assembly of the lipid microtubules. Trehalose (18.9 mg/mL) was added to the microtubule solution and then dehydrated using a rotary evaporator.

For the individual treatments, 1 mg of dehydrated microtubules were loaded with BDNF (Millepore, Temecula, CA), CA-Cdc42, or CA-Rac1 (Rho GTPases conjugated to TAT were purified in Dr. Susann Brady-Kalnay's Laboratory) and the loading concentrations were 500 μg/mL, 500 μg/mL and 230 μg/ml, respectively. The protein

loaded microtubules were mixed into 2.6% agarose (w/v) at a 1:1 volume ratio. For the combination treatments, each protein was loaded into 2 mg of microtubules at the same concentration as in the individual groups. The protein loaded microtubules were mixed into the 2.6% SeaPrep® agarose at a volume ratio of 1:1:2 for BDNF:Rho GTPase:agarose. This was to ensure that the same concentration of total protein delivered in the individual treatments was injected in the combination groups.

4.2.4. Characterization of Microtubule Mediated Sustained Release of BDNF I*n Vitro*

Eighty microliters of 500 μg/mL BDNF was loaded into 2 mg of lipid microtubules by incubating the BDNF solution with dehydrated microtubules. The BDNF loaded microtubules were mixed into 2.6% SeaPrep® agarose to obtain a microtubule concentration of 8.33 mg/mL in 1.3% concentration of agarose. Forty microliters of the BDNF loaded microtubules and agarose mixture was added into wells of a 96 well plate, allowed to gel, and 150 μL of PBS was added to the above mixture. The 96 well plate was placed in a 37°C oven and after the first 24 hours, the 150 μL of PBS was removed and replaced with 150 μL of fresh PBS. This method was followed every 48 hours for the following 13 days. The retrieved PBS solutions were stored in -20°C until they were ready to be analyzed for BDNF content. The BDNF released from the microtubules was quantified using the BDNF ELISA Sandwich Kit (Chemicon). Briefly, the BDNF standards and the PBS solutions containing BDNF collected over 14 days were allowed to attach onto a precoated 96 well plate overnight. The standards and BDNF experimental wells were treated with monoclonal anti-BDNF for 2.5 hours. Then the second antibody streptavidin-HRP was added to the wells for 1 hour.

Tetramethylbenzidine (TMB) was added to each well to stain for the secondary antibody. Lastly, the standard and sample wells were placed in the Synergy HT Micro Detection Microplate Reader (BIO-TEK Instruments, Inc., Winooski, VT) and the absorbance was read at 450 nm. A standard curve was obtained from the absorbances of the BDNF standards to determine the amount of the BDNF released in the PBS.

4.2.5. Conjugation of Rhodamine to BDNF

To permit visualization and characterization of BDNF's diffusion through the spinal cord tissue, Rhodamine was conjugated to BDNF. The protocol provided with the EZ-Label™ Rhodamine Protein Labeling Kit (Pierce, Rockford, IL) was followed to conjugate the Rhodamine to BDNF (BDNF/Rhodamine). Briefly, Rhodamine was added to 1 mg/mL of BDNF at a 5-fold molar excess. The Rhodamine and BDNF solution was incubated for 1 h at 25 °C. The solution was collected and then dialyzed in 0.1 M PBS for 1 h to remove unconjugated Rhodamine. The BDNF/Rhodamine was diluted to 500 μg/mL and then loaded to the microtubules as mentioned above. The agarose/BDNF/Rhodamine loaded microtubules mixture was injected into the spinal cord as described below. After 2 weeks the spinal cords were retrieved and processed, which is described in further detail below.

4.2.6. Cooling System for *In Situ* Gelling Agarose

Our laboratory has previously established that SeaPrep® agarose was supportive of neurite extension *in vitro* and regeneration *in vivo*. However, as this form of hydroethylated agarose gels at 17 °C, a cooling system was developed to enable rapid,

localized cooling at the lesion site for *in situ* gelation. The system consisted of tubing that ran from the regulator on a nitrogen gas tank into a Styrofoam box (4300 cm^3) that contained a 100% ethanol/dry ice, which served to lower the temperature of the gas. The tubing then came out of the box and at the end was an aluminum rod that released the cooled nitrogen gas (Fig. 4.1). Surrounding the aluminum rod was a 50 mL tube that contained dry ice to maintain the low temperature. The cooled nitrogen gas was applied over the agarose filled spinal cord cavity for 30 seconds to ensure that the agarose gelled.

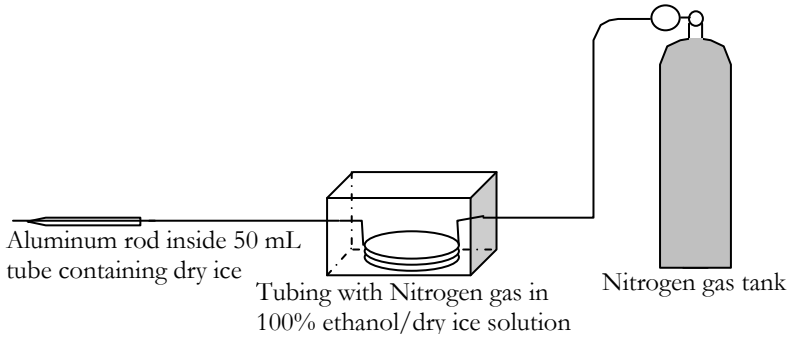


Figure 4.1. Gel cooling system. Tygon tubing ran from the regulator on the nitrogen gas tank to a Styrofoam box that contained a 100% ethanol/dry ice solution and then ran out of the tube and ended with an aluminum rod inside a 50 mL tube that contained dry ice to maintain the low temperature. The cooled nitrogen gas was directed over the agarose filled spinal cord cavity to cause *in situ* gelation of the agarose solution.

Surgical Procedures

4.2.7. Implantation of Agarose-Protein Scaffolds in a Dorsal Over-hemisection Model *In Vivo*

Male Sprague-Dawley (Charles River Laboratory) rats weighing 190-230 grams were anesthetized with Nembutal®. The skin and muscle were opened on the back to expose the thoracic vertebrae T8-T10. Fine rongeurs were used to remove the bone and

expose the spinal cord. A modified dorsal over-hemisection was made removing 2 mm × 2 mm × 1.5 mm deep spinal cord section at T10, removing the dorsal portion of the CST. The protein loaded microtubules/agarose solution was injected using a 1-10 μL pipet, into the spinal cord cavity as mentioned above in the section describing the fabrication and loading of the microtubules. The cooling system was used to gel the agarose scaffold and the agarose scaffold containing the microtubules loaded with protein as mentioned above. The cooled nitrogen gas was applied over the agarose filled spinal cord cavity for 30 seconds to ensure that the agarose gelled. After the scaffold was injected into the cavity, the muscle was sutured together and the skin was closed with wound clips. The animals were manually expressed twice a day until urinary function was recovered.

4.2.8. Anterograde Neuronal Tracer Injection into the Corticospinal Tract

Four weeks post-injury, biotinylated dextran amine (BDA, Invitrogen) was bilaterally injected into the motor cortex to trace the axons in the CST. The animals were anesthetized with 2% isoflurane and maintained through the surgery at 0.5% isoflurane. The skin and the periosteum were opened to expose the skull. Three injection sites, located within each hemisphere 2 mm from the midline and 1 mm apart from each site, were used to inject 0.5 μL of BDA at each site over 2 min. Six weeks post-injury, the animals were anesthetized with ketamine (1 mL/kg), xylazine (0.17 mL/kg), and Acepromazine (0.37 mL/kg), transcardially perfused with 4% paraformaldehyde, the spinal cords from T9-T11 were retrieved, and incubated in 4% paraformaldehyde overnight and then stored in PBS containing 0.01% sodium azide.

4.2.9. Immunohistochemistry and Histological Evaluation of Explanted Spinal Cords and Hydrogels

The spinal cords were transferred from PBS to 30% sucrose and allowed to incubate overnight. Serial sagittal cryostat (Leica CM 300, Leica, Bannockburn, IL) sections of 25 μm thickness were made of the spinal cords and mounted onto glass slides. Immunohistochemical staining for reactive astrocytes and microglia/macrophages, as well as growth inhibitory extracellular matrix molecules, such as CSPGs, was conducted. In addition, to help visualize the axons and neurons at the injury site, neurofilament staining was conducted as described below.

A triple-stain was performed on the spinal cord sections to visualize anti-rabbit glial fibrillary acidic protein (GFAP) (Chemicon), ED-1 (mouse anti-rat CD68, Serotec, Raleigh, NC), and BDA. The sections were incubated in 4% goat serum in 0.5% Triton-X 100 for 1 hour. GFAP helps identify reactive astrocytes, while ED-1 stains for reactive microglia and macrophages. GFAP (1:1000 dilution) and ED-1 (1:1000 dilution) in 4% goat serum and 0.5% Triton-X 100 were added to the sections and incubated at 4°C overnight. The sections were washed three times with 0.5% Triton-X 100 and then the secondary antibodies, Alexa Fluor 350 goat anti-rabbit IgG (1:200 dilution) (Molecular Probes, Eugene, OR), Alexa Fluor 594 goat anti-mouse IgG_1 (1:200 dilution) (Molecular Probes), and Alexa Fluor 488 Streptavidin (1:200 dilution) were added for GFAP, ED-1, and BDA, respectively, and allowed to incubate for 1 hour at room temperature. The sections were rinsed thrice with 0.5% Triton-X 100 and twice with 0.1M PBS and then the slides containing the spinal cord sections were mounted with glass coverslips.

A triple stain was also done with GFAP and CS-56, an antibody to identify growth inhibitory CSPGs (Sigma, St. Louis, MO), along with BDA. Similar to the method mentioned above for the GFAP/ED-1/BDA stain, the sections were incubated in 4% goat serum in 0.5% Triton-X 100 for 1 hour. The GFAP (1:1000 dilution) and CS-56 (1:250 dilution) were added to the 4% goat serum in 0.5% Triton-X 100, placed over the spinal cord sections, and was incubated in 4 °C overnight. The sections were washed three times with 0.5% Triton-X 100 and then the secondary antibodies, Alexa Fluor 350 goat anti-rabbit IgG (1:200 dilution), Alexa Fluor 594 goat anti-mouse IgM (1:200 dilution), and Alexa Fluor 488 Streptavidin were added to the sections. Glass coverslips were placed on top of the sections.

A double stain with BDA and anti-mouse Neurofilament 160 kDa (NF-160, Sigma), used to stain for the neurofilament of the neurons and axons, was performed. Similar to the method mentioned above, the sections were incubated in 4% goat serum in 0.5% Triton-X 100 for 1 hour. The NF-160 (1:500) was added to the 4% goat serum in 0.5% Triton-X 100, placed over the spinal cord sections, and was incubated at 4 °C overnight. The sections were washed three times with 0.5% Triton-X 100 and then Alexa Fluor 594 goat anti-mouse (1:200 dilution) to stain for neurofilament and Alexa Fluor 488 Streptavidin were added to the sections for 1 hour at room temperature. The slides containing the spinal cord sections were mounted with coverslips and images were taken on the Zeiss Axioskop 2 Plus microscope (Zeiss, Thornwood, NY).

4.2.10. Quantitative Analysis of Cellular, Molecular, and Axonal Response to Scaffold and Protein

Methodology for GFAP and CS-56 Analysis

The methodology to quantify the fluorescent intensity of GFAP and CS-56 images has been previously published (Jain et al. 2006). Briefly, to quantify the cellular response to the SCI, the immunostained images (i.e., GFAP and CS-56) were captured with a Olympus digital camera. The intensity of the immunostains were quantified, averaged and compared using MATLAB (Mathwork) based image analysis program. This program generated line profiles (30 line profiles/image) and extracted relative fluorescent intensity as a function of distance from the interface between the agarose gel filled cavity and spinal cord tissue. Four images/spinal cord section were taken at the interface (Fig. 4.2A). All images were taken at the same exposure time and conditions.

For each stained image, a reference arc was generated at the interface and then 30 radial line profiles were generated (Fig. 4.2B). An average intensity profile for each staining (i.e., GFAP and CS-56) was calculated by averaging the coinciding increments of the 30 line profiles and displayed by an average intensity profile versus distance from the interface. A minimum of 60 images (4 images/spinal cord section x 5 spinal cord section/each animal x 3 animals) per experimental group was utilized to obtain the overall average intensity profiles for each staining representing 1800 different individual intensity profiles per condition. The overall average intensity profiles for both GFAP and CS-56 as a function of distance from the interface was compared between experimental groups.

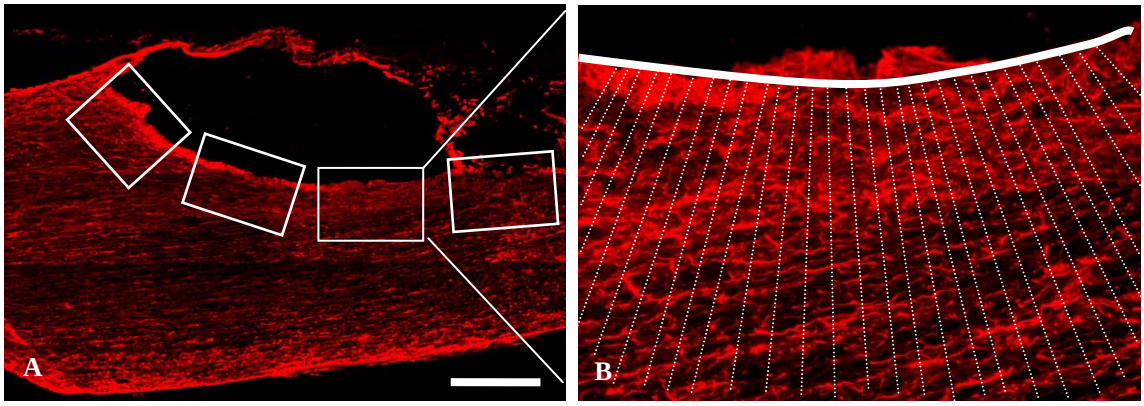


Figure 4.2. Method for quantitative analysis of immunostained spinal cord sections. **A.** 10x immunostained image with four boxes made along the spinal cord/scaffold interface, which will be used for quantitative analysis. Scale bar = 500 μm **B.** One of the four boxes from A that was enlarged (20x immunostained image) to analyze the fluorescent intensity. The arc represents the interface and the 30 radial lines will be generated using a custom designed MATLAB program and then averaged to display an average intensity profile for GFAP, CS-56, and NF-160 versus the distance from the interface.

Quantification of ED-1$^+$ Cells

To determine if the hydrogel/microtubule delivery system elicited a chronic inflammatory response, the number of ED-1$^+$ cells was quantified in and around the lesion site. Two 10x images were taken of each section using an Olympus digital camera attached to the Zeiss Axioskop 2 Plus microscope. ImagePro software (Media Cybernetics, Carlsbad, CA) was used to count the number of cells that were present. Two parameters, (1) the fluorescent intensity and (2) cell diameter, were kept constant for all the images of the different conditions when counting the cells.

4.2.11. Quantification of Average Lesion Area

The lesion area was measured to determine whether the scaffold and delivered proteins influenced the size of the lesion site after SCI. Sections that were located at the

epicenter of the lesion were used to determine the lesion area. The average lesion area was then calculated for each of the conditions.

4.2.12. Axonal Outgrowth Through Inhibitory Regions Proximal to Lesion Site

CSPGs are present in the glial scar around the lesion area. However, it was observed that there were CSPG regions proximal to the lesion site not part of the glial scar. The CSPG regions were identified by CS-56 staining. A region was considered to be inhibitory if the fluorescent intensity values were similar to the intensity values in the glial scar. The BDA^+ axons were placed in three categories: (1) axons that stopped before the inhibitory region, (2) axons passed the proximal interface of the inhibitory region; however, stopped within the inhibitory region, and (3) passed the distal interface of the inhibitory region; and were counted (Fig. 4.3). A percent of axons in each region was determined by the following equation:

(No. of axons in specific region/ Total number of axons in all 3 regions) *100

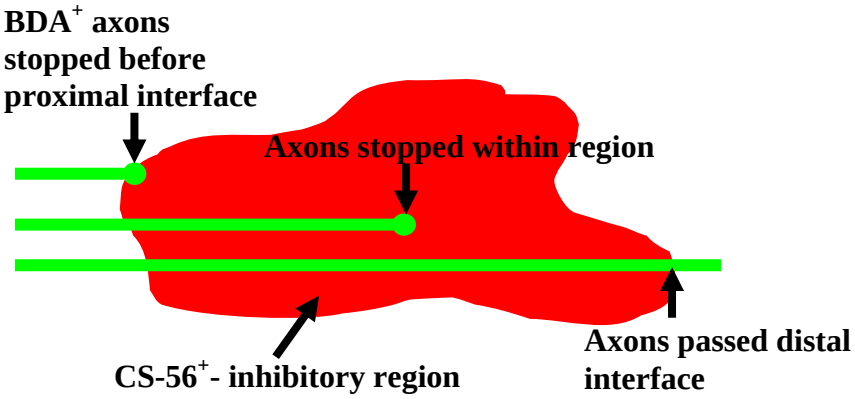


Figure 4.3. Schematic of axon quantification in $CS\text{-}56^+$-inhibitory regions. Axons were placed into 3 categories: 1) axons that stopped before the proximal interface of the inhibitory region, 2) axons that stopped within the inhibitory region, and 3) axons that passed the distal interface of the inhibitory region.

Distance Traveled by Axons within Inhibitory Region

The axons that had stopped within the inhibitory region mentioned in the section above were then measured to determine the distance traveled after crossing the proximal interface. The shortest total distance traveled by the axons within the inhibitory region was measured and then averaged. The length of all the axons that stopped their outgrowth within this region was measured and then averaged for each condition. The percent of distance traveled by the axons within the CS-56 was calculated by the following equation:

(Average Axonal Distance Traveled/Total Length of Inhibitory Region) *100

4.2.13. Quantification of BDA^+ Axons

Spinal cord sections stained for BDA^+ axons were imaged at 10x on the Nikon Eclipse 80i upright microscope using a Microfire CCD camera (Optronics, Goleta, CA) that interfaced with the Neurolucida software (MicroBrightField Bioscience, Williston, VT) to obtain a montage of each section. The images were used to measure axonal retraction from the lesion site for all of the conditions. The distance from the 3 or more closest axons to the lesion site was measured using ImagePro Software.

The beginning of the proximal side of the lesion was marked as 0 mm and every millimeter was marked up until 4 mm rostral to the lesion site (Fig. 4.4). The number of BDA^+ axons, from 0 to 1 mm, 1 to 2 mm, 2 to 3 mm, and 3 to 4 mm, proximal to the injury site was counted. The number of axons 4 mm proximal to the lesion site was considered as the total number of axons. The percent of axonal outgrowth was determined by the following equation:

(Number of axons at specific distance range/Total number of axons at 4 mm) * 100

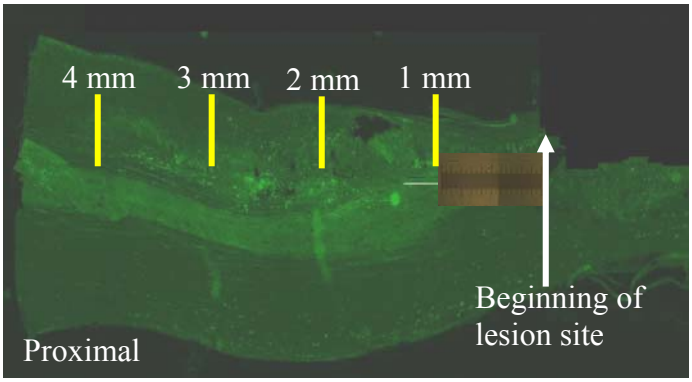


Figure 4.4. Schematic for quantification of the percentage of BDA^+ axons from beginning of the lesion site to 4 mm proximal.

4.2.14. Behavioral Analysis

Behavioral tests were performed to determine if there was any functional improvement in the control or treated animals. The grid walk and the beam walk tests were performed at 3 and 5 weeks post-injury. During the grid walk tests, the number of slips, where the hind feet slipped through the wires in the grid, was counted over 2 min. For the beam walk test, the animals walked a beam that was 5 cm in width and 105 cm in length. The number of slips of the hind feet was counted.

4.2.15. Statistical Analysis

GFAP, CS-56, and NF 160: The area under the curve of the overall average intensity profiles was determined for four different bins: 0 to 100 μm, 100 to 200 μm, 200 to 300 μm, and 300 to 400 μm from the interface extending into the spinal cord. The

area under the curve of the profiles was statistically compared by ANOVA and Tukey's test ($P < 0.05$). To determine if there was significant difference amongst the conditions and between different groups, ANOVA and Tukey's test ($P < 0.05$) was performed for all other analysis.

4.3. RESULTS

4.3.1. Characterization of Agarose Hydrogels

Rheology was used to determine the gelling temperature for various concentrations of SeaKem®, SeaPlaque®, and SeaPrep® agarose. As the temperature decreases, the viscous modulus, also referred to as loss modulus, of the agarose increases. It can be seen that the average gellation temperature for the various concentrations for SeaKem®, SeaPlaque®, and SeaPrep® were 35, 25, and 11°C, respectively. When E9 chick DRGs were cultured *in vitro* in agarose gels in the presence of exogenous NGF, the 1% SeaPrep® agarose scaffolds supported robust neurite extension in 3D compared to the various concentrations of SeaKem® and SeaPlaque® (Fig. 4.6).

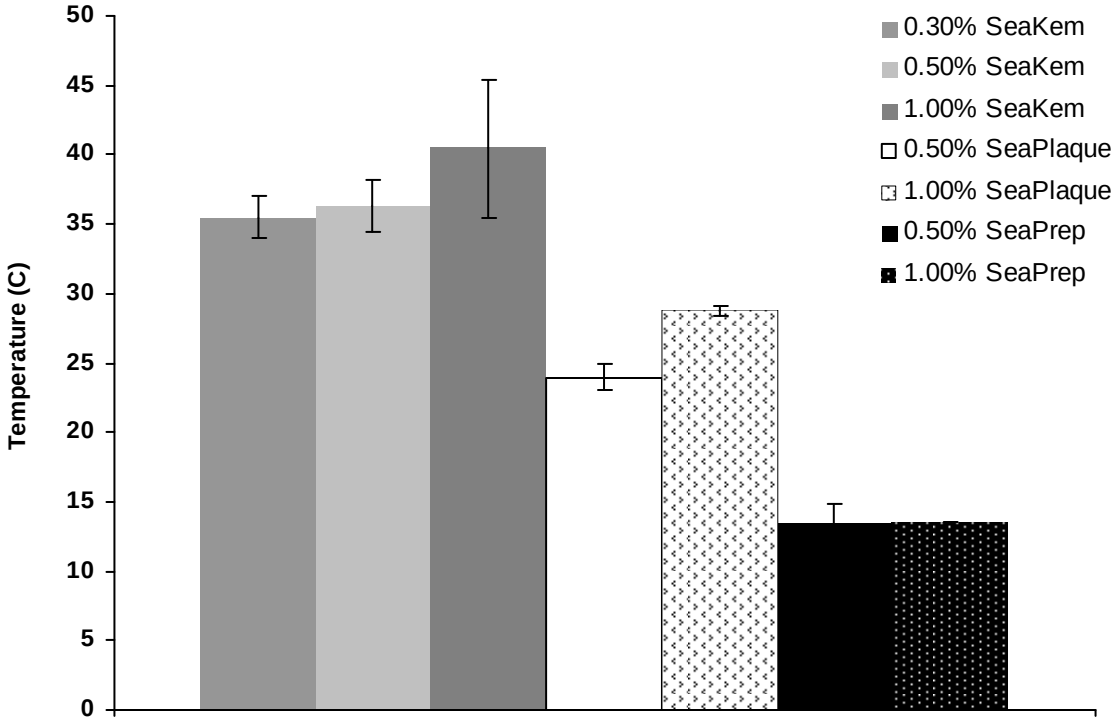
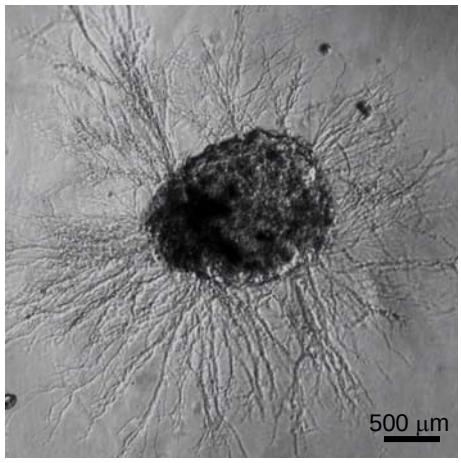
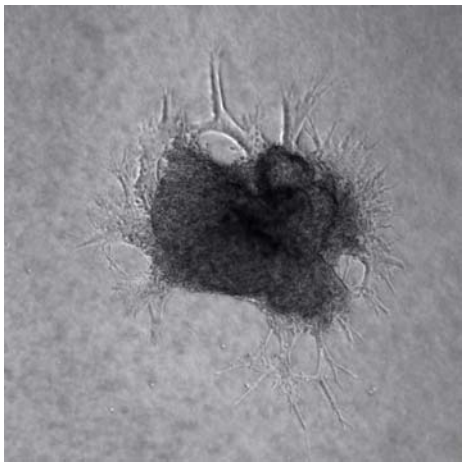


Figure 4.5. Gelling temperatures for various agarose gels and concentrations. The average gelling temperature for SeaKem® agarose was around physiological temperatures for the various concentrations. The various concentrations of SeaPlaque® agarose gels approximately at 26°C. SeaPrep® agarose gels approximately at 11 °C. The data represent the mean ± SEM.





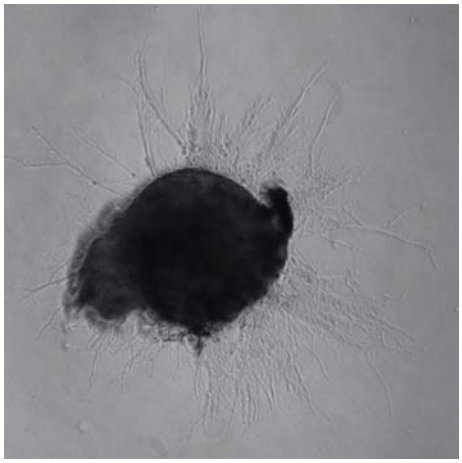


Figure 4.6. Micrograph images of DRGs cultured in different agarose gels after 48 hrs at 5x magnification. **A.** 1% SeaPrep® agarose. **B.** 0.5% SeaPlaque® agarose. **C.** 0.3% SeaKem® agarose. Greater number and longest neurite outgrowth was in the 1% SeaPrep® agarose

4.3.2. Characterization of BDNF Release

An *in vitro* release assay for BDNF loaded microtubules was performed. Approximately 3 μg of BDNF was released within the first 24 h. A cumulative of 4 μg of BDNF was released within the first 3 days. In Figure 4.7B, it is shown that approximately 30 ng/day of BDNF was released for the subsequent 11 days. Microtubules loaded with BDNF/Rhodamine embedded in the agarose were injected into the spinal cord cavity. After two weeks, the spinal cords were retrieved and sectioned. After two weeks of delivery, BDNF/Rhodamine can be seen in the spinal cord tissue between 1 to 2 mm proximal to the lesion site. (Fig. 4.7C). An ED-1 stain for macrophages/microglia and DAPI stain for nuclei were performed to observe if there was co-localization with BDNF/Rhodamine. It can be seen that some of the BDNF/Rhodamine co-localized with ED-1^+ cells and DAPI (Fig. 4.7D and E).

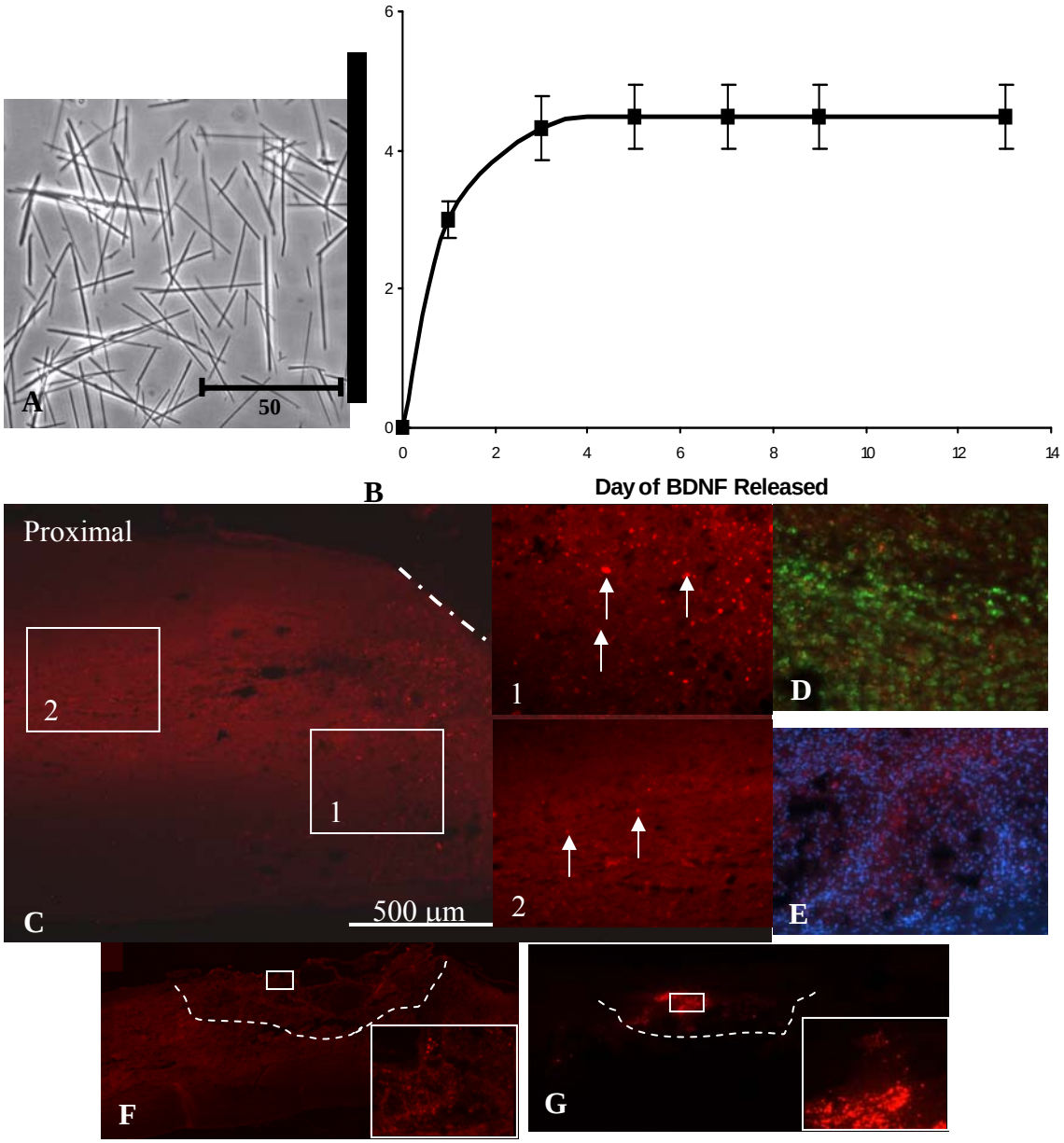


Figure 4.7. Diffusion of BDNF. **A**. Bright field image of lipid microtubules. **B**. *In vitro* release assay of BDNF over the first 2 weeks. The graph shows that an initial burst released 4 µg of BDNF within the first 3 days. An average of 30 ng/day of BDNF was released for the following 11 days. The data represent mean ± SEM **C.** An image of the proximal region of a spinal cord section after delivery of BDNF/Rhodamine at 4x. The 20x images labeled 1 and 2 are outlined with white boxes in C and demonstrate that BDNF/Rhodamine diffused approximate 2 mm proximal to the lesion site. The dashed line represents the interface between the spinal cord and scaffold. White arrows indicated BDNF/Rhodamine. **D.** Image of ED-1^{+} cells and BDNF/Rhodamine (red) in 10x. BDNF can be seen co-localized with ED-1^{+} stain, as well as in the tissue. **E.** Image of DAPI (blue) and BDNF/Rhodamine (red) in 20x. BDNF can be seen co-localized with DAPI, as well as in the tissue. **F and G.** BDNF/Rhodamine in scaffold 48 hrs and 1 week post-injury at 10x. White box represents focused inset image.

4.3.3. *In Situ* Gelling Hydrogel in the Spinal Cord Cavity

The liquid phase of SeaPrep® agarose was injected into the spinal cord cavity. Cooled nitrogen gas was applied above the cavity containing the injected hydrogel until the agarose had gelled. It can be seen in Figure 4.8 that the agarose gelled in the spinal cord cavity.

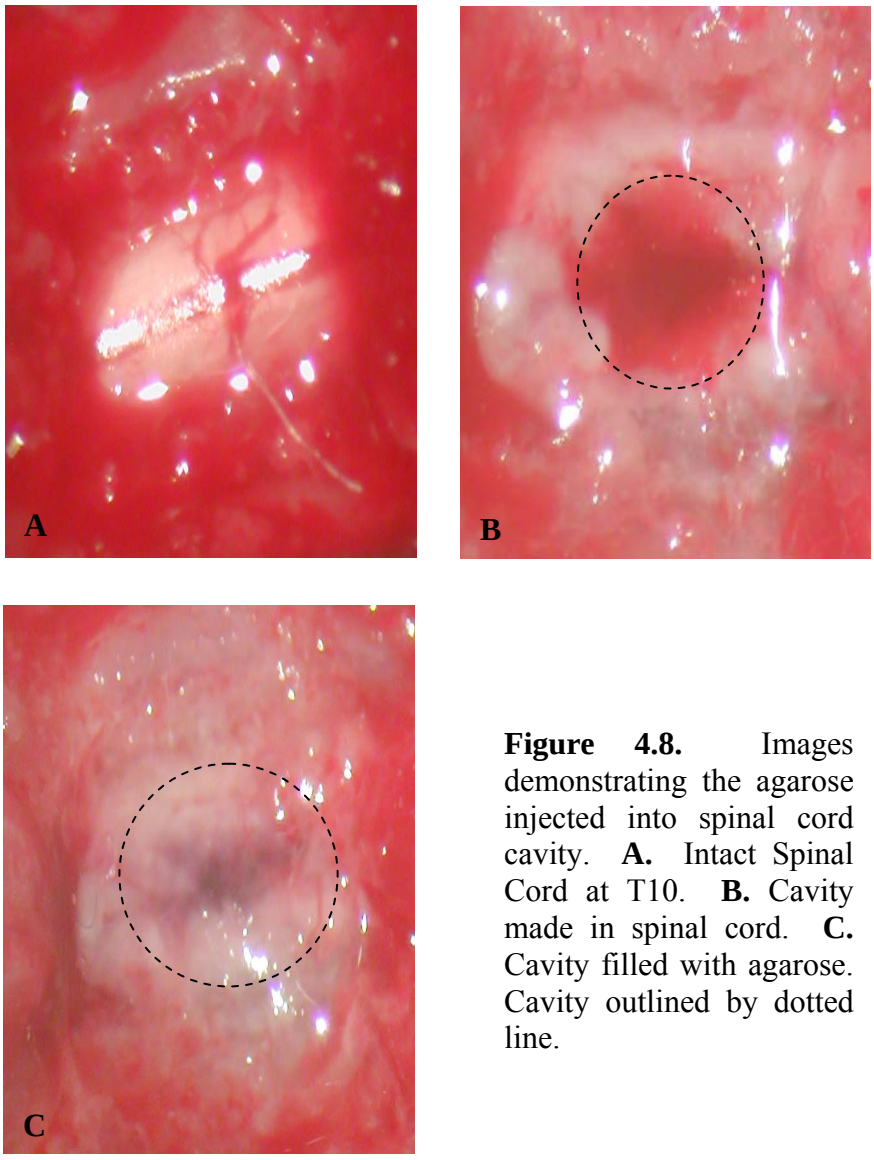


Figure 4.8. Images demonstrating the agarose injected into spinal cord cavity. **A.** Intact Spinal Cord at T10. **B.** Cavity made in spinal cord. **C.** Cavity filled with agarose. Cavity outlined by dotted line.

4.3.4. Cellular and Molecular Inflammatory Response

The inflammatory response after injury was measured by observing cellular and molecular response. Spinal cord sections were incubated with antibodies to GFAP, ED-1 and CS-56, which stained for astrocytes, microglia/macrophages, and CSPGs, respectively. In Figure 4.9, micrograph images of reactive astrocytes response and CSPG deposition at the interface between the spinal cord cavity and the implants can be seen. The regions with high GFAP intensity generally correlates with increased CS-56 intensity. A custom MATLAB program was developed to measure and correlate how the fluorescent pixel intensity changed from the scaffold/spinal cord interface radially outward into the spinal cord. The graphs in Figures 4.10 and 4.11, demonstrate that the GFAP and CS-56 intensities are lower for CA-Cdc42, CA-Rac1, and BDNF as compared to the untreated control. Also, CA-Cdc42 condition had significantly lower GFAP and CS-56 intensity compared to the agarose control and fluorescent intensity was statistically lower for BDNF treatment compared to the agarose control.

Besides staining for GFAP and CS-56 for the inflammatory response at 6 weeks, reactive microglia/macrophages were quantified by counting the number of ED-1^{+} cells in and around the lesion site. A statistical difference between the two control conditions, untreated and agarose groups, and the experimentally treated animals was not observed (Fig. 4.12).

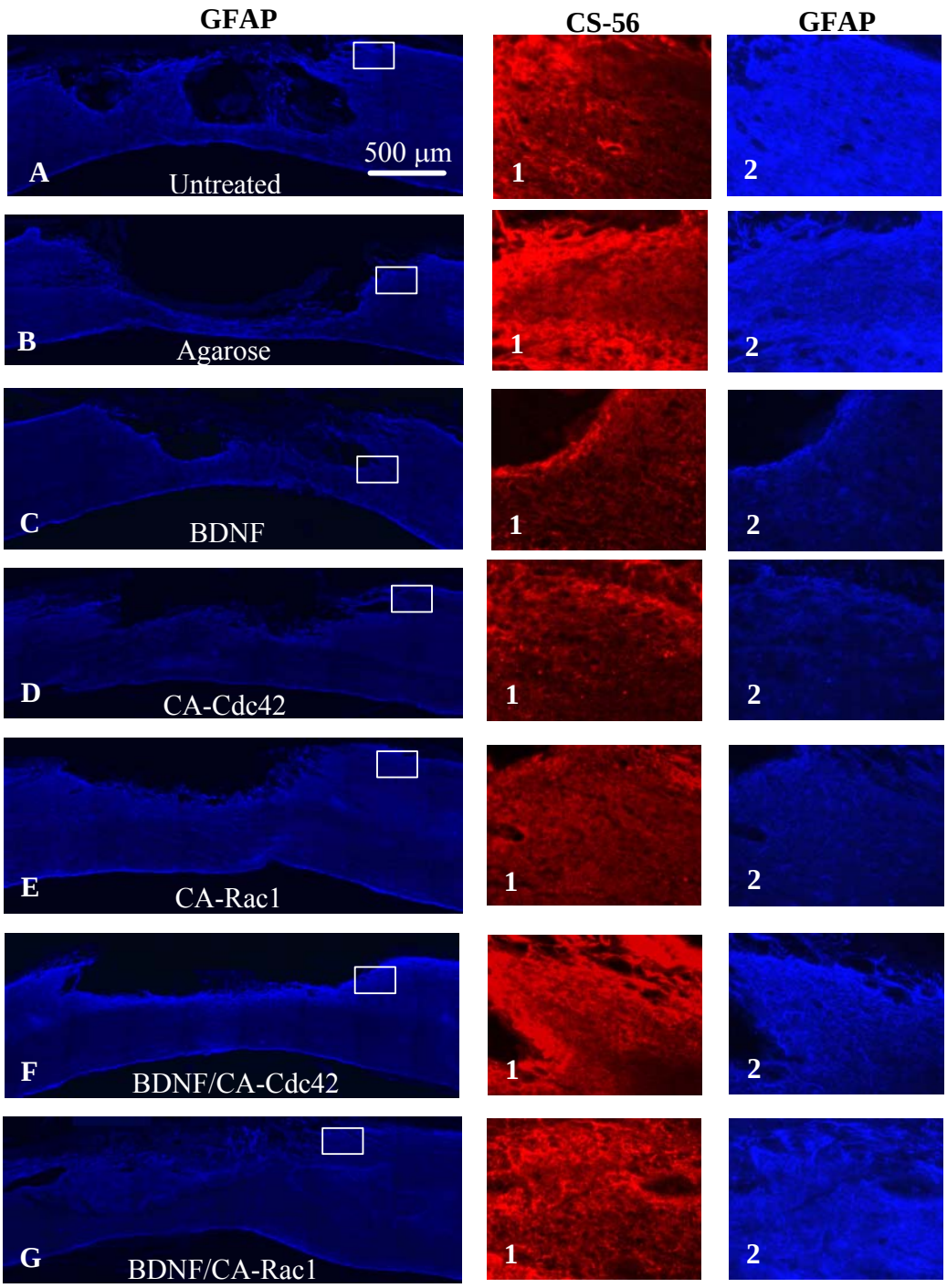
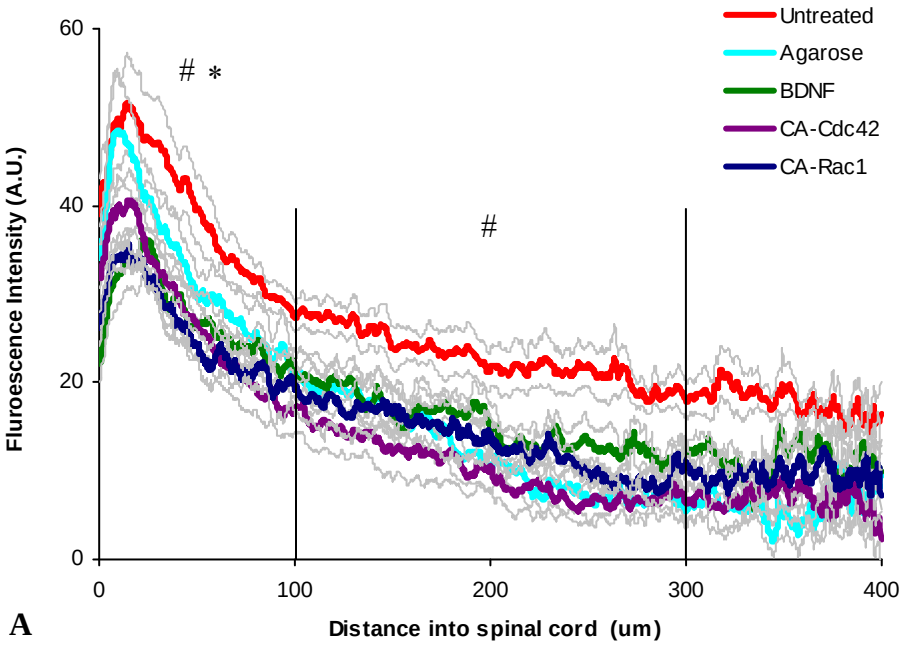
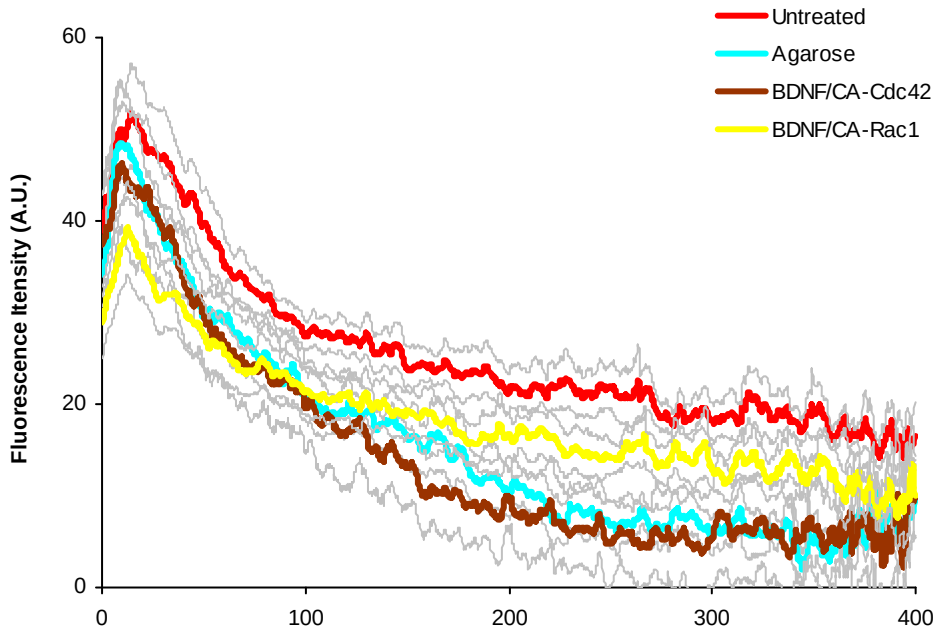


Figure 4.9. GFAP and CS-56 expression at the lesion site. **A-G.** Images of the lesion site for the controls and treated conditions that show the GFAP stain for reactive astrocytes at 10x. The white box represents the focused image (20x) to the right of CS-56 (red) and GFAP (blue) in 1 and 2, respectively. **A.** Untreated spinal cord. **B**. Agarose **C.** BDNF treated animals. **D**. CA-Cdc42 **E.** CA-Rac1 **F.** BDNF/CA-Cdc42 **G**. BDNF/CA-Rac1.



A



B

Figure 4.10. GFAP fluorescent intensity from the interface into the spinal cord. **A.** Individual treatments. The intensity for BDNF,CA-Cdc42, and CA-Rac1 compared to the untreated control is significantly less. CA-Cdc42 was significantly less intense compared to the agarose control. **B.** Combination treatments. A significant difference was not observed compared to the controls. The data represent the mean. The gray lines represent the ± SEM. (# $p < 0.05$ BDNF, CA-Cdc42, and CA-Rac1 compared to untreated control, * $p<0.05$ CA-Cdc42 compared to agarose control).

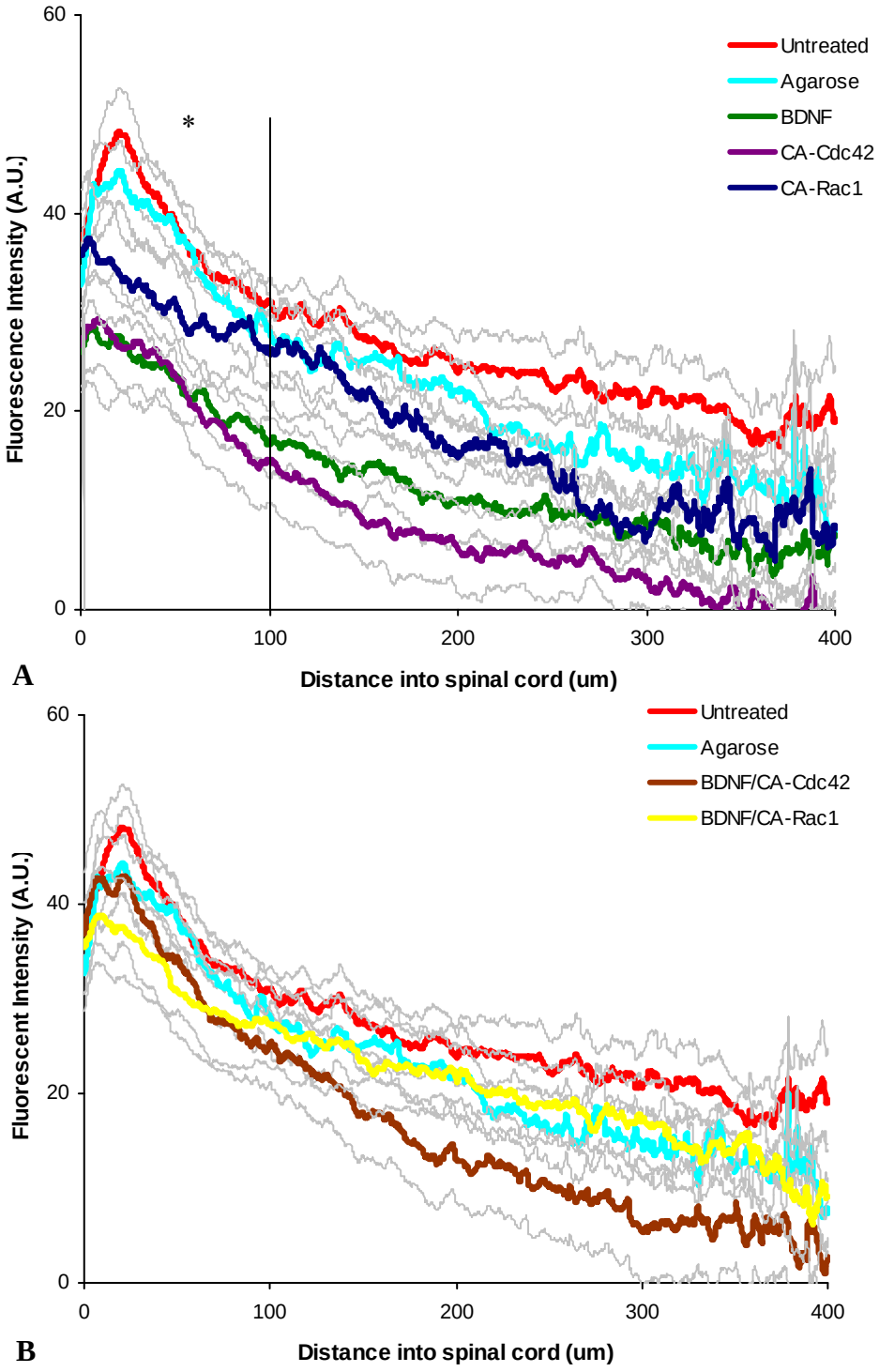


Figure 4.11. CS-56 fluorescent intensity from the interface into the spinal cord. **A.** Individual treatments. The CS-56 intensity was significantly lower in spinal cords treated with CA-Cdc42 and BDNF compared to the untreated and agarose controls. **B.** Combination treatments. The data represent the mean. The gray lines represent ± SEM. (*$p<0.05$ CA-Cdc42 and BDNF compared to untreated and agarose controls).

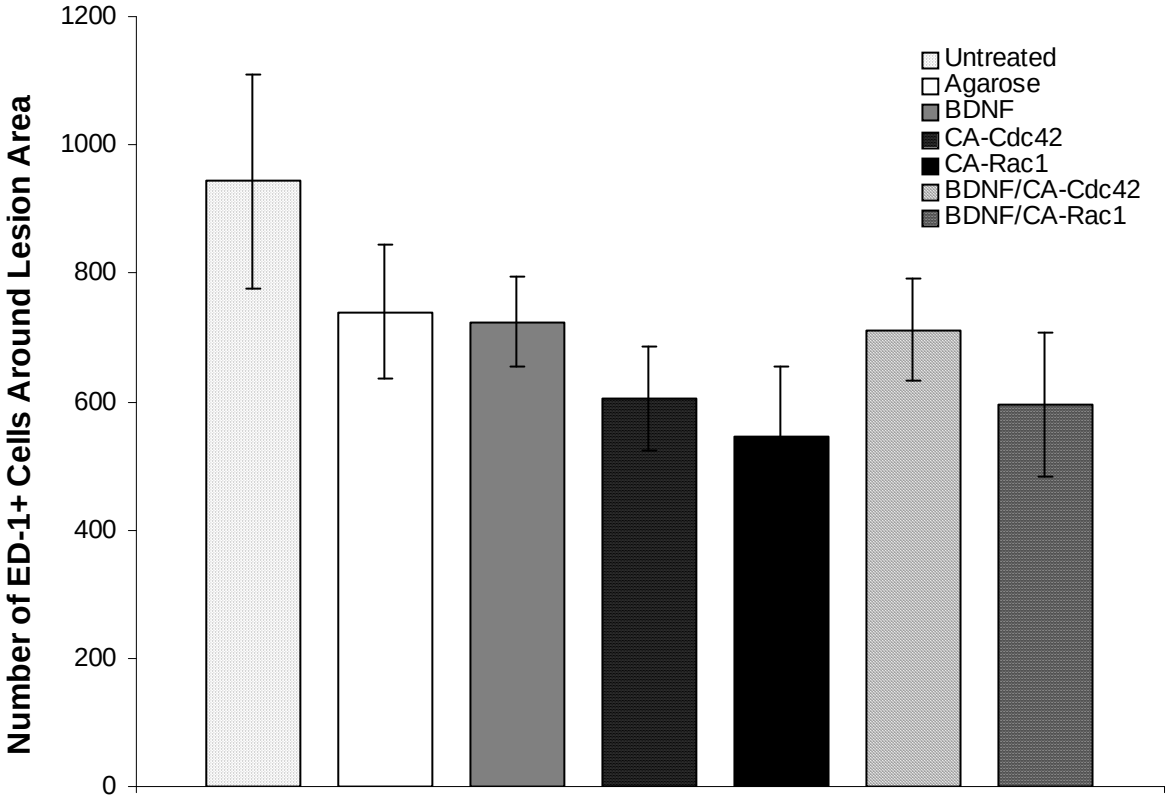


Figure 4.12. Comparison of the number of ED-1^{+} macrophages/reactive microglia around the lesion area. This bar graph shows that there was no statistical difference in the number of macrophages/reactive microglia in the treated animals compared to the untreated and agarose controls. The data represent the mean ± SEM.

The average lesion area was measured for all the experimental groups to determine if the treatments caused a greater secondary injury. The average lesion area for the untreated and agarose controls was greater than 1 mm^2, which was significantly larger than all of the treated groups, BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 that had an area approximately of 0.7 mm^2 (Fig. 4.13).

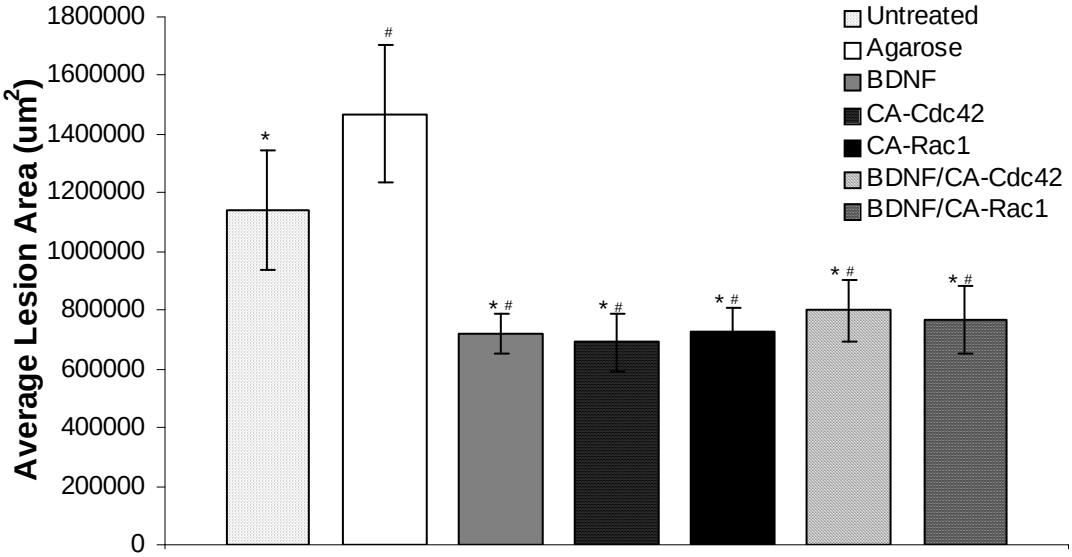


Figure 4.13. Average lesion area of the controls and treated spinal cords. The average lesion area was statistically smaller in all of the treated conditions compared to the untreated and agarose controls. The data represent the mean ± SEM. (* $p<0.05$ compared to untreated control and #$p<0.05$ compared to agarose control).

4.3.5. Crossing of Axons through Inhibitory Regions *In Vivo*

CS-56^{+} regions were identified proximal to the lesion site, aside from the glial scar in the treated and control groups. The area was considered to be inhibitory if the CS-56 pixel intensity values were within the same range as those in the glial scar expressed in the immediate vicinity of the lesion. The fluorescent images of the controls and treated conditions can be seen in Figure 4.14. The number of axons that crossed the region, stopped within the inhibitory region, or stopped proximal to the inhibitory region interface was counted. The percentage of axons that stopped in each of the 3 regions was determined (Fig. 4.15A). The untreated and agarose animals had a significantly higher number of axons that stopped before the proximal interface of the inhibitory region compared to the animals treated with CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1. All the treated groups had axons that traversed the distal interface of the inhibitory region, which was significantly higher than the control groups. However, in all the conditions, a high percentage of axons, 45-65%, stopped within the inhibitory region (Fig. 4.15A).

Due to the high percentage of axons that stopped within the inhibitory region shown in Figure 4.15A, the distance the axons extended from the proximal interface and stopped within the inhibitory region was measured relative to the size of the inhibitory region. The percent of distance extended by the axons was determined by measuring the average axonal distance traveled by the total distance of the possible path. It can be seen that the controls, untreated and agarose, extended less than half the distance of the CS-56 intense region (Fig. 4.15B). However, CA-Rac1 and BDNF/CA-Rac1 had axons that extended at a significantly higher percent of distance, over 60%, than the controls.

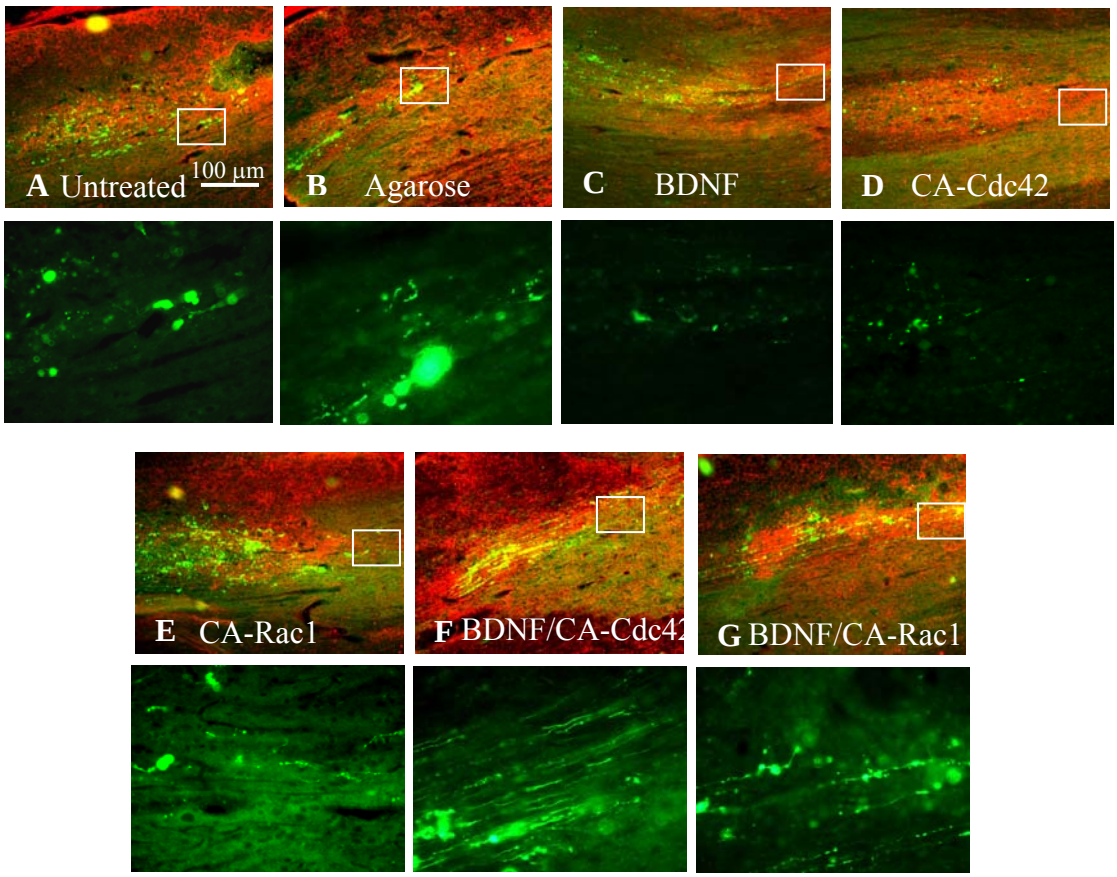


Figure 4.14. BDA^{+} axons and CS-56^{+} inhibitory regions. A-G are 10x images of BDA and CS-56. The white box represents the image below (40x) of BDA. **A.** Untreated. **B.** Agarose. **C.** BDNF. **D.** CA-Cdc42. **E.** CA-Rac1. **F.** BDNF/CA-Cdc42. **G.** BDNF/CA-Rac1. The control groups have a high percentage of axons that have stopped before or within the inhibitory regions. However, the treatment groups have a significantly higher percentage of axons that crossed the distal interface of the inhibitory region compare to the untreated and agarose controls.

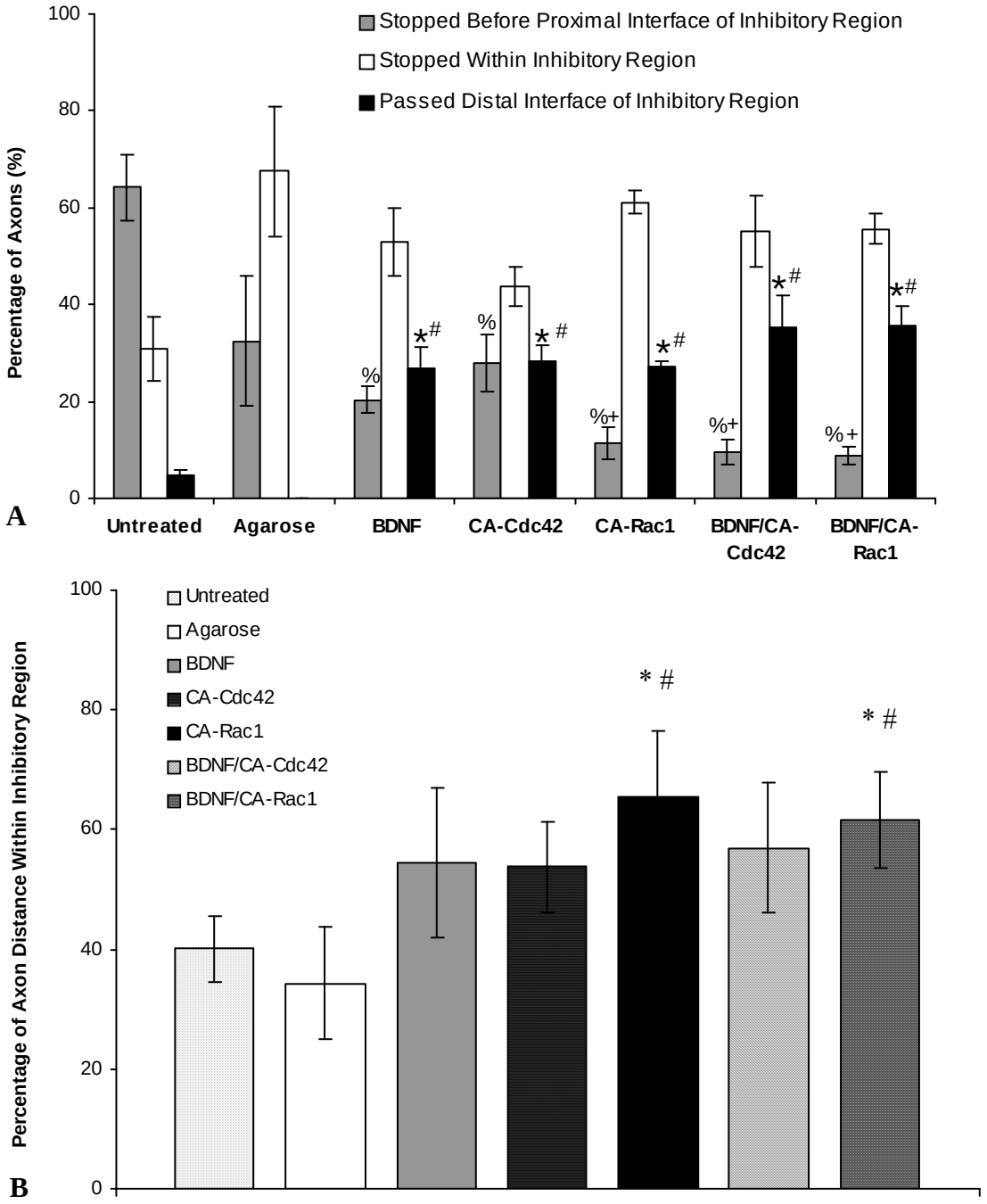


Figure 4.15. Percentage of axons in CS-56^{+} inhibitory regions. **A.** A significantly higher percentage of axons crossed the distal interface of the inhibitory region in the BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 compared to the untreated and agarose controls. Also, the axons in the controls stopped at the proximal interface of the inhibitory region at a significantly higher percentage than in the spinal cords treated with CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1. **B.** In the spinal cords treated with CA-Rac1 and BDNF/CA-Rac1, the axons traveled a significantly further distance within the inhibitory region than the untreated and agarose controls. (* and % $p<0.05$ compared to untreated control and # and + $p<0.05$ compared to agarose control). The data represents mean ± SEM.

4.3.6. Sprouting Axons after Implantation of Hydrogel/Protein-Microtubule Delivery System

BDA^+ axons in the CST were measured to quantify the distance of retraction from the lesion site. The beginning of the proximal side of the lesion was marked as the origin and the distance to the closest axons (3 or more axons) were measured. The micrographs and the graph (Figs. 4.16 and 4.17) show that the axons in the two controls, untreated and agarose conditions, retracted over 1 mm from the beginning of the lesion site. However, the treated groups had axons within 1 mm of the beginning of the lesion site, more specifically; the axons were approximately 400 μm away from the lesion site in the groups treated with BDNF, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1. Thus, there was significant difference amongst BDNF, CA-Rac1, BDNF/CA-Rac1, and BDNF/CA-Cdc42 from the controls.

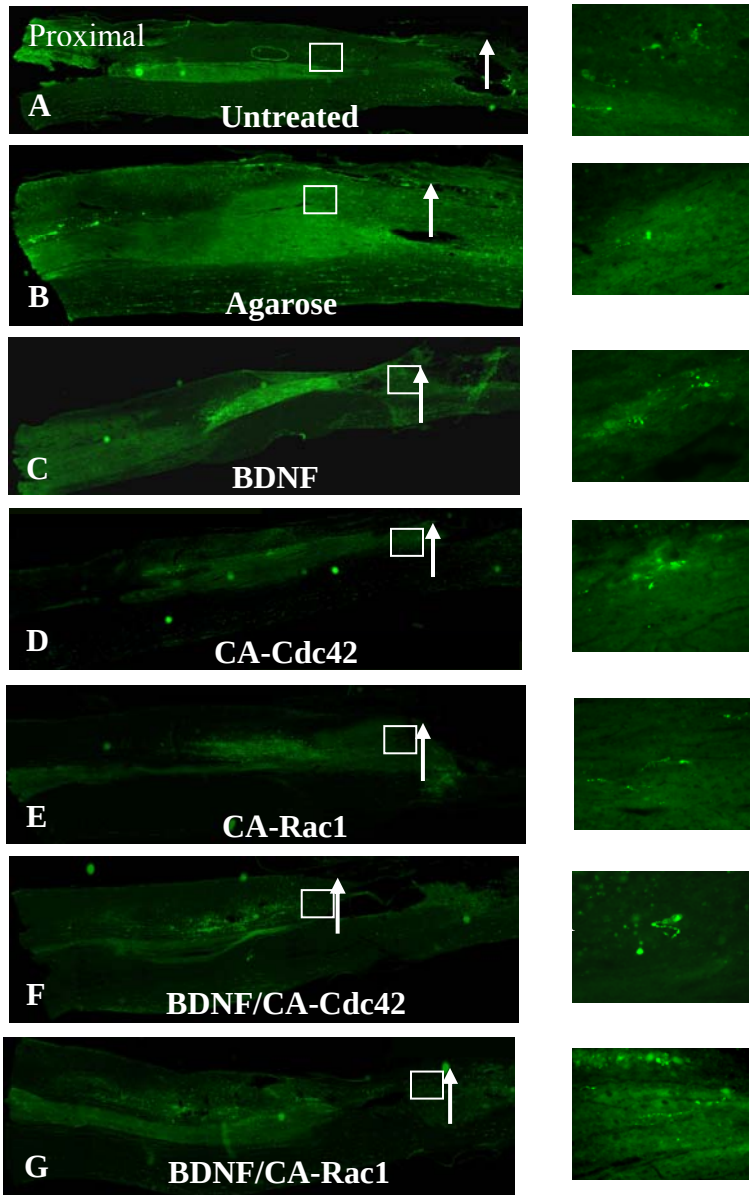


Figure 4.16 BDA^{+} axons in CST. Fluorescent images of controls and treated conditions at 10x. White box represents image to the right in 40x. **A.** Untreated **B.** Agarose **C.** BDNF **D.** CA-Cdc42 **E.** CA-Rac1 **F.** BDNF/CA-Cdc42 **G.** BDNF/CA-Rac1 The arrows represent the begging of the lesion site. The images on the right show the closest axons to the lesion site.

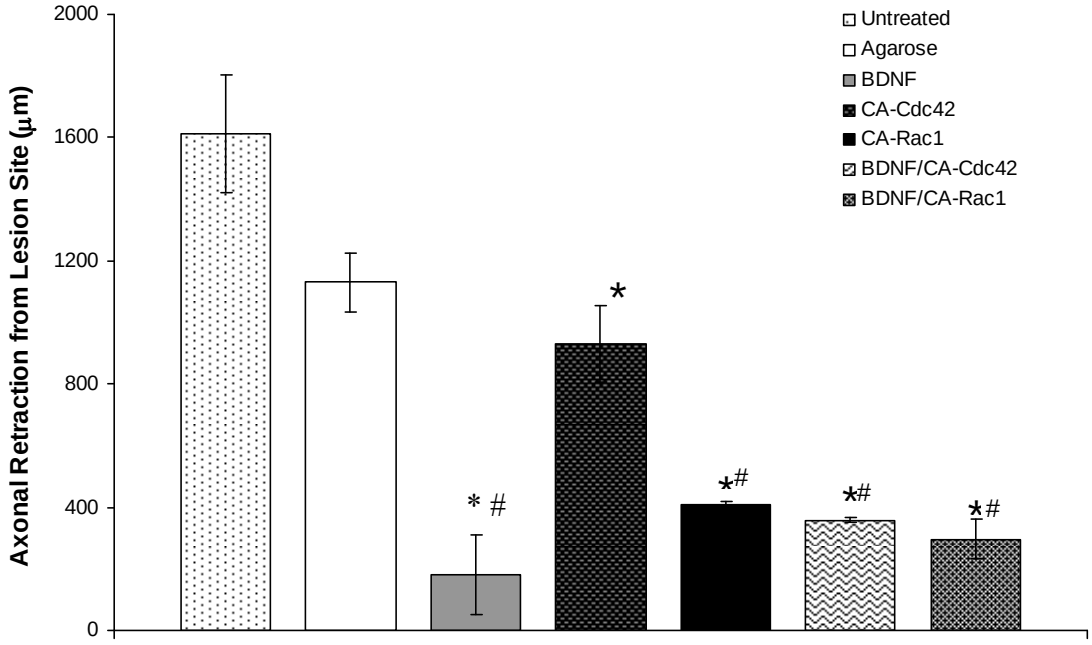


Figure 4.17. Axonal retraction from the lesion site in the treated and control spinal cords. The distance of 3 or more axons from the lesion site in each image were averaged. All the treated cords were within 1 mm of the lesion site and were significantly closer to the lesion site compared to the untreated cords. BDNF, CA-Rac1, BDNF/CA-Cdc42, BDNF-CA-Rac1 were statistically closer to the agarose control and were within 400 μm to the lesion site. The data represents mean ± SEM. (* $p<0.05$ compared to untreated controls and # $p< 0.05$ compared to agarose control).

Besides measuring the distance from the lesion site to the closest axons, the number of axons was quantified between the lesion site and 4 mm proximal and the percent of axonal outgrowth towards the lesion site was determined. The lesion site was labeled as the origin, 0 mm, and the proximal side was marked every millimeter, up to 4 mm (Fig. 4.4). The proximal side of the spinal cord was divided into 4 regions of 0 to 1 mm, 1 to 2 mm, 2 to 3 mm, and 3 to 4 mm. The axons were counted in each of the regions. The number of axons that were present at 4 mm was considered to be the total number of axons because axons stopped retracting before that distance proximal to the lesion site. It can be seen that the percent of axons for the two controls as the axons extended closer to the lesion site decreased and were significantly lower than the number of axons for the CA-Rac1, BDNF/CA-Rac1, and BDNF/CA-Cdc42 (Fig. 4.18). By 2 mm, there were twice the percentage of axons in the treated groups, CA-Rac1, CA-Cdc42, BDNF/CA-Rac1, and BDNF/CA-Cdc42 compared to the untreated and agarose controls. Between the ranges of 0 to 1 mm, there was significant difference in the number of axons between the treatment groups and the controls.

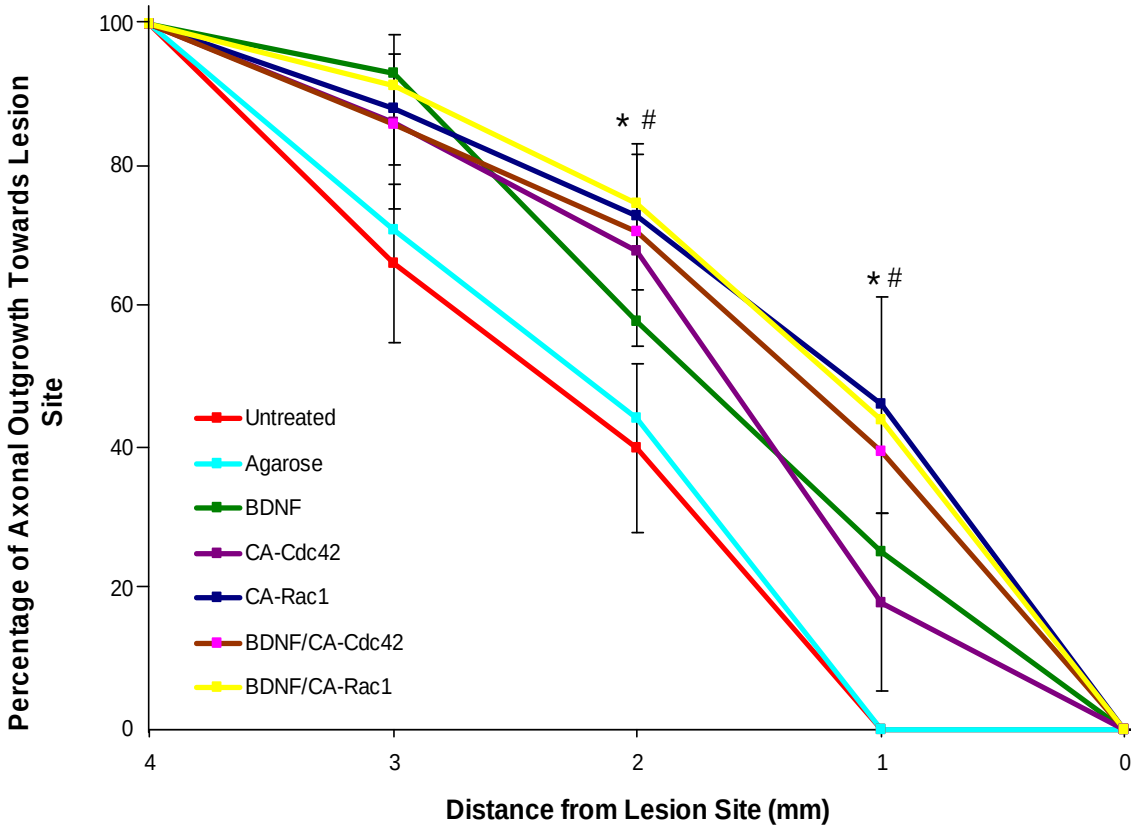


Figure 4.18. Percent of axonal outgrowth towards the lesion site. The percent of axons was measured from 4 mm proximal to the beginning of the lesion site. The graph shows that 2 mm from the lesion site, the 75-80% of the axons are present in the conditions treated with CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 compared to the untreated and agarose controls, which had significantly lower percent of axons (45-50%). One millimeter away from the lesion site, there were not any axons in the control conditions, where as there were 45-50% of the axons in the spinal cords treated with CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1. The data represents mean ± SEM (* $p<0.05$ compared to untreated control and # $p<0.05$ compared to agarose).

Another method to observe axonal outgrowth was to stain the spinal cord sections against NF-160, which can be seen in the micrographs (Fig. 4.19). The NF-160 stain was quantified using the custom MATLAB program utilized for the GFAP and CS-56 analysis; however, rather than quantifying the pixel intensity from the interface into the spinal cord, the fluorescent intensity was measured from the interface into the scaffold/spinal cord cavity. In the untreated and agarose controls, the NF-160 staining had significantly less intensity compared to the treated conditions (Fig. 4.20). The axons in the control groups did not infiltrate a significant distance into the scaffold, about 150 μm. However, compared to the controls, the treatment groups, BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1, had a significantly higher fluorescent intensity compared to the controls and the intensity levels did not change with distance into the scaffold. The treatment groups had statistically traveled further through the hydrogel scaffold than the untreated and agarose controls. Although there was statistical difference between the controls and the treatment groups, a significant difference amongst the treatment groups was not observed.

4.3.7. Behavioral Analysis

Behavioral tests, grid and beam walk were performed to determine if functional improvement occurred. The tests were performed 3 and 5 weeks post-injury. A statistical difference amongst the controls and the treatment groups was not observed (Fig. 4.21).

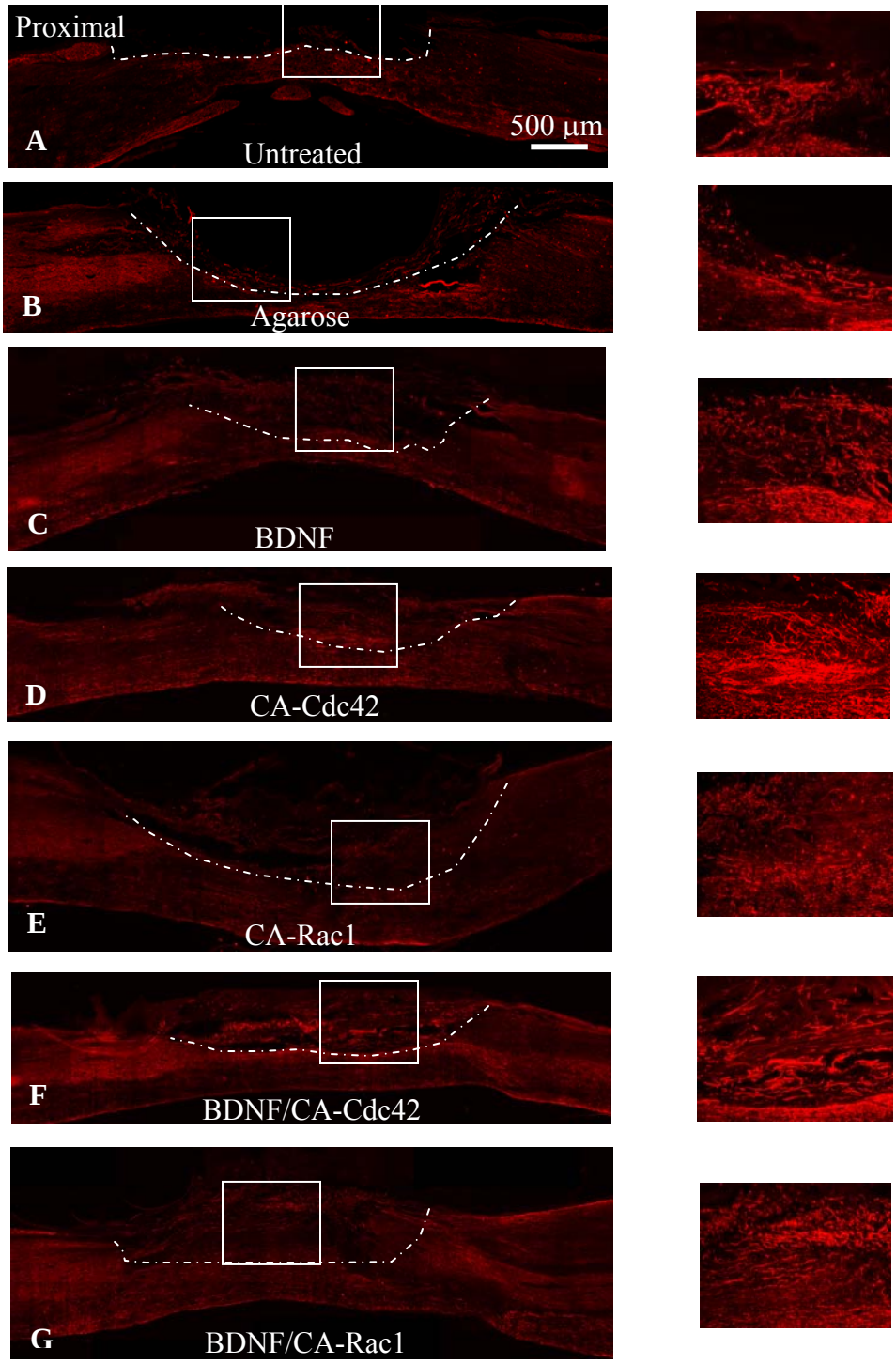


Figure 4.19. NF-160^{+} Fluorescent Images. **A-G.** Representative images for the controls and treated conditions with NF-160 stain at 10x. The interface between the spinal cord tissue and scaffold is identified with a white dashed line. To the right of each image, is a 10x image of the area outlined with a white box. **A**. Untreated. **B.** Agarose **C.** BDNF **D.** CA-Cdc42. **E.** CA-Rac1. **F.** BDNF/CA-Cdc42. **G.** BDNF/CA-Rac1. In C-G, the treated conditions had NF-160^{+} axons in the scaffold-filled cavity, where as NF-160^{+} axons were not observed in the controls.

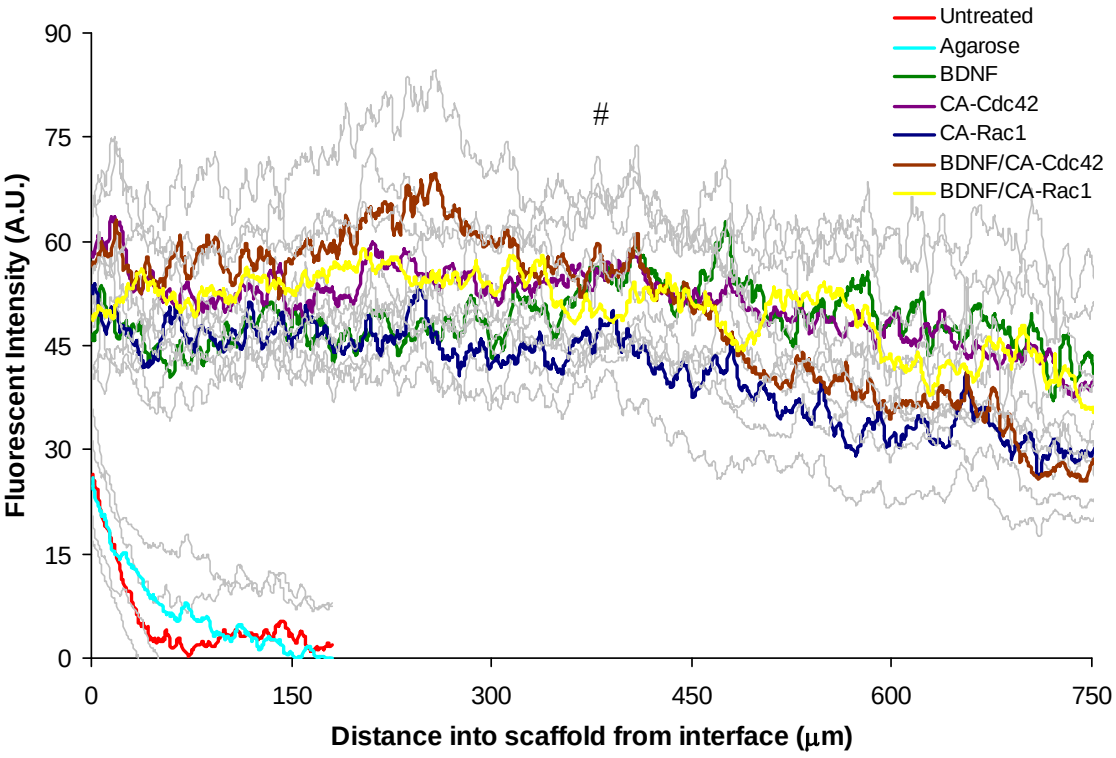


Figure 4.20. Quantitative analysis of NF-160 intensity for the stained spinal cords. The spinal cords treated with CA-Cdc42, CA-Rac1, BDNF, BDNF/CA-Cdc42, and BDNF/CA-Rac1 had significantly higher fluorescent intensity and also had extended further into the scaffold-filled spinal cord cavity than the untreated and agarose controls. Gray lines represent ± SEM (# $p<0.05$ treatment groups compared to untreated and agarose controls).

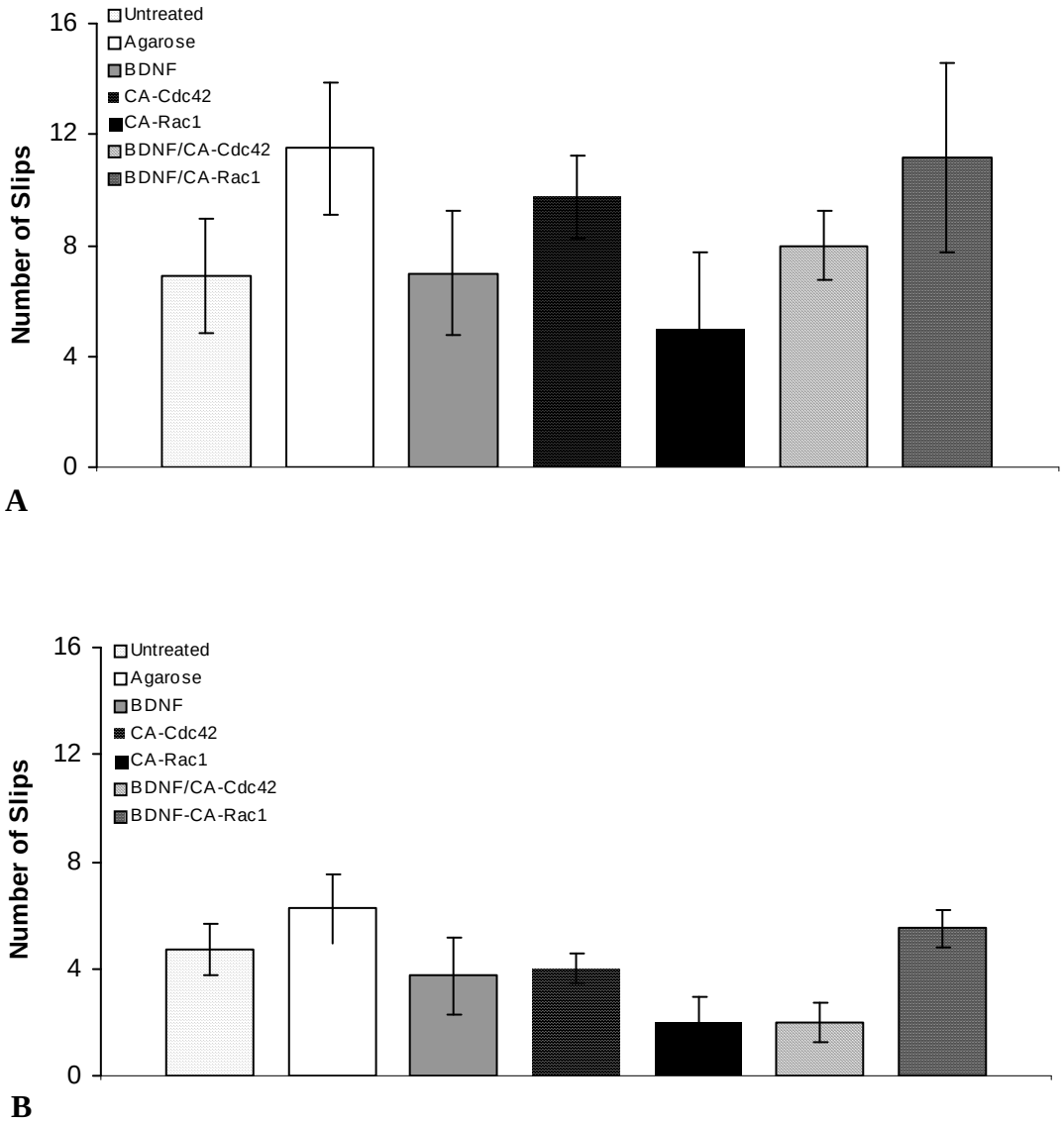
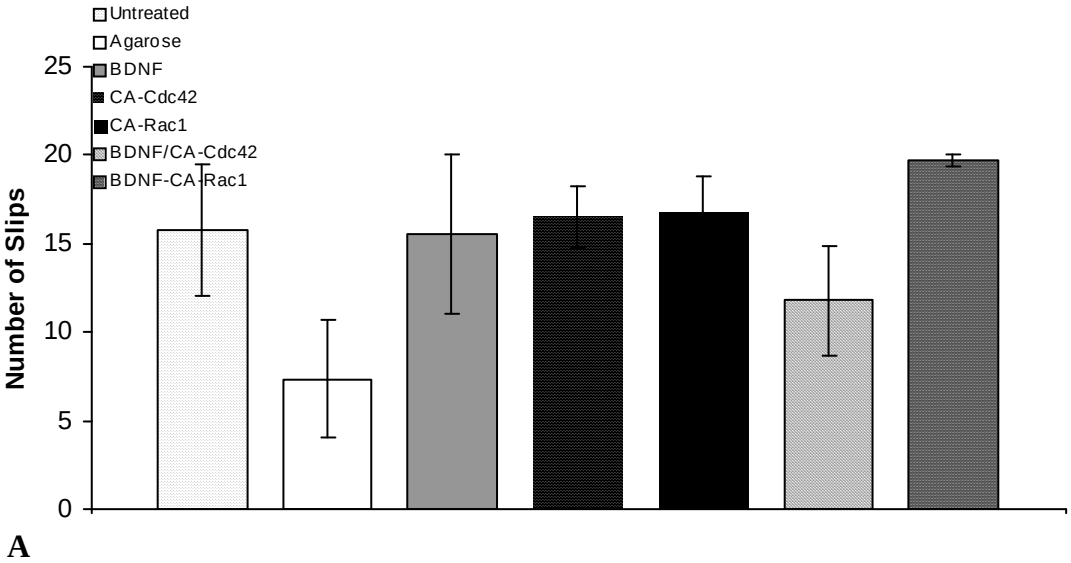
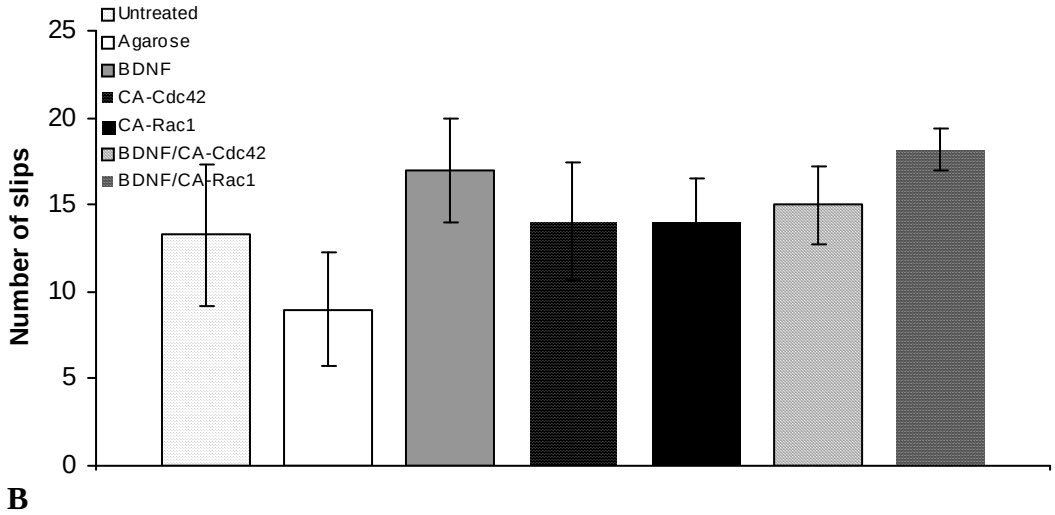


Figure 4.21. Grid walk test at 3 and 5 weeks post-injury. **A.** Grid walk test at 3 weeks. **B.** Grid walk test at 5 weeks. A statistical difference amongst the groups for either 3 or 5 weeks, was not observed. There was also no significant functional improvement when comparing the same condition at 3 and 5 weeks. The data represent the mean ± SEM.



A



B

Figure 4.22. Beam walk test at 3 and 5 weeks post-injury. **A.** Beam walk test at 3 weeks. **B.** Beam walk test at 5 weeks. A statistical difference amongst the groups for either 3 or 5 weeks, was not observed. There was also no significant functional improvement when comparing the same condition at 3 and 5 weeks. The data represent the mean ± SEM.

4.4. DISCUSSION

This study was performed to determine whether BDNF, CA-Cdc42, CA-Rac1, delivered individually or in combination utilizing the hydrogel/microtubule scaffold delivery system would encourage axonal outgrowth through the CSPG-inhibitory regions and influence the inflammatory response, particularly the reactivity of the astrocytes and CSPG deposition. The results demonstrated that the individual treatments, BDNF, CA-Cdc42, and CA-Rac1 reduced the $GFAP^+$ and $CS\text{-}56^+$ regions in the glial scar around the lesion site. It was also exhibited that the treatment conditions promoted axonal outgrowth through CSPG-rich inhibitory regions proximal to the lesion site. Although BDA^+ axons did not infiltrate the scaffold-filled cavity, a significantly higher percentage of axons were present within 1 mm of the lesion site compared to the untreated and agarose controls. The growth permissivity of the hydrogel in the lesion cavity was demonstrated by the high number of $NF\text{-}160^+$ sprouted fibers within the scaffold.

The ideal 3-D scaffold that supports and promotes axonal outgrowth across a lesion in the spinal cord remains elusive. It is important to minimize the physical gap between the spinal cord and the scaffold to make the microenvironment more conducive for growth, as well as minimize any inflammatory response. One advantage of utilizing the agarose is its ability to gel *in situ* and conformally fill the defect by adopting its shape. To investigate whether the agarose would provide an adequate scaffold, we utilized the dorsal-over hemisection model, which transects the dorsal portion of the CST.

Initially, *in vitro* experiments were performed to characterize the temperature sensitive gelling properties of agarose hydrogels to explore their suitability for gelation *in vivo* and to characterize neurite extension in 3D agarose cultures. Using rheology, the

specific temperature range at which the agarose changes from liquid phase to a gel phase was determined to be at physiological temperatures, 35 to 37.5 °C for SeaKem®, 22.5 to 27 °C for SeaPlaque®, and 11 °C for SeaPrep® (Fig. 4.5). Another important feature is that the *in situ* gelling hydrogels are capable of supporting neurite extension *in vitro*. This characteristic of the hydrogel is important as it suggests that should regenerating fibers be enticed to enter the gel, the scaffold would provide a supportive substrate for neurite extension. The *in vitro* experiments demonstrated that 1% SeaPrep® supported the greatest amount of neurite outgrowth. Also, the SeaPrep® agarose gel has been well characterized by our laboratory *in vitro* (Balgude et al. 2001; Dillon et al. 1998) and *in vivo* in the peripheral nervous system (Yu and Bellamkonda 2003), demonstrating that hydrogels are capable of supporting neurite extension *in vitro*. This characteristic of the hydrogel is important as it suggests that should regenerating fibers be enticed to enter the gel, the scaffold would provide a supportive substrate for neurite extension.

The agarose gelling *in situ* within the spinal cord defect ensures that it adopts the shape of the cavity, thus minimizing the gap between the spinal cord tissue and the agarose. Several studies have reported inserting hydrogels, such as NeuroGel™ (Woerly et al. 2001; Woerly et al. 2004), alginate (Kataoka et al. 2004), and collagen (Kataoka et al. 2004) between the nerve gaps, however; the hydrogels were either inserted as a sponge or as a pre-formed gel. When filling irregular defects, we suggest that *in situ* gelling is critical as it is difficult to 'pre-fabricate' a gel of the exact shape and dimensions of an irregular soft tissue defect *in vivo*. However, as the agarose gel that is optimal for neurite extension gels at the low temperature of 11 °C, we developed the hydrogel cooling system that can cool agarose in liquid phase at the lesion site rapidly

(within seconds). When evaluating the ability of our gels to conformally fill the defect, there existed a possibility that the agarose would leak out of the cavity/defect generated by hemisection of the spinal cord before it gelled. However, upon retrieval and processing of the spinal cord, it was observed that the agarose scaffold did in fact gel *in situ* and was restricted within the lesion site.

As evident by visual inspection and histological inspection, the explanted agarose scaffolds remained in the spinal cord lesion for 6 weeks. No shrinkage of the gel was observed, and the gel-spinal cord interface appears stable after six week *in vivo* confirming the performance of the agarose gels as a viable *in situ* gelling biopolymer.

To encourage axonal outgrowth into the hydrogel scaffold, we embedded lipid microtubules that released CA-Cdc42, CA-Rac1, and BDNF, individually or in combination, in a sustained manner within the agarose gel construct. The sustained release of BDNF was characterized *in vitro* and *in vivo*. The *in vitro* release assay demonstrated that BDNF was released from the microtubules for at least 2 weeks (Fig. 4.7B). BDNF was conjugated to Rhodamine and delivered using the hydrogel/microtubule delivery system. Two weeks post-implantation, the spinal cords were explanted. It can be seen that BDNF/Rhodamine is present around the lesion site, as well as up to 2 mm proximal to the lesion site (Fig. 4.7C). These sections were double stained with antibody against ED-1 and complete co-localization was not observed between the ED-1^{+} cells and delivered BDNF/Rhodamine (Fig. 4.7D). We believe that the delivery of the Rho GTPases will demonstrate similar characteristics due to the molecular weight of BDNF and the Rho GTPases being approximately 25 kDa. This is a crucial time period due to the formation of the glial scar and severed axons retracting

away from the lesion site. Therefore, delivery of the proteins during this time period will affect the inflammatory response and axonal regrowth due to the proteins being transduced into microglia, macrophages, astsrocytes, as well as neurons. Other data from our laboratory have demonstrated that we have the capability to optimize the microtubules to release bioactive agents over a period of 5-7 weeks if necessary (Meilander et al. 2003; Meilander et al. 2001). The microtubules release proteins through diffusion; and have well characterized the release profile, which provided the information on the loading concentration to ensure the release of the therapeutic concentration (10-100 ng/ml).

The Rho GTPases, Cdc42 and Rac1, were delivered in their constitutively active form, as well as BDNF, to encourage axonal outgrowth through the inhibitory regions after injury. *In vitro* studies of Cdc42 and Rac1 and *in vivo* studies of BDNF have demonstrated that these proteins affect the actin cytoskeleton dynamics, thereby influencing axonal outgrowth. Antibodies against GFAP and CS-56 were used to stain spinal cord sections to observe the affects of these three proteins delivered individually and in combination on reactive astrocytes and CSPG deposition.

The quantification of the GFAP and CS-56 fluorescent pixel intensities demonstrated that the spinal cords treated with CA-Cdc42, CA-Rac1, and BDNF have significantly lower expression of GFAP and CSPGs than the untreated control; however, the combination treatments, BDNF/CA-Cdc42 and BDNF/CA-Rac1 had similar intensities to the controls. As mentioned above, it has been established that Cdc42 and Rac1 are involved in actin cytoskeleton dynamics. In a study investigating the migration of astrocytes in an *in vitro* wound healing response, it was demonstrated that inhibition of

Rho by C3, a bacterial toxin, increased the astrocytes' migratory response (Holtje et al. 2005), evidenced by GFAP expression. In this study, when CA-Cdc42, CA-Rac1, and BDNF were delivered individually, these exogenous proteins may have triggered a feedback mechanism activating an alternate signaling pathway, causing the astrocytes to become less migratory. For the combination treatments, it has been shown that BDNF and Cdc42 converge on the same pathway affecting the actin cytoskeleton, with BDNF upstream on the transduction pathway (Chen et al. 2006; Yuan et al. 2003). Therefore, it is possible that the delivery of CA-Cdc42 and BDNF in combination, for the concentrations delivered, caused feedback inhibition, activating an alternate pathway due to a higher concentration of Cdc42, including exogenous CA-Cdc42, possibly resulting in increased astrocyte migration.

In another study, Y27632, an inhibitor to Rho Kinase, which is a downstream effector of Rho, was delivered to the spinal cord after a dorsal column transection. It was shown that GFAP expression increased in the Y27632 treated animals around the lesion cavity (Chan et al. 2007). The CST axons did not infiltrate the glial scar. In the same study, *in vitro* co-cultures of astrocytes treated with Y27632 and cortical neurons demonstrated increased expression of CSPGs (identified by CS-56 staining); however, Y27632 did not inhibit neurite outgrowth. This demonstrates that although GFAP expression had increased, the potential for axonal outgrowth into the glial scar had increased, as well. Similar results were observed for GFAP expression in our combination studies. The axonal retraction was less pronounced in the BDNF/CA-Cdc42 and BDNF/CA-Rac1 treated groups compared to the controls; however, GFAP expression was similar to the untreated and agarose controls. This phenomenon did not

occur in the individual treatments; therefore, it is possible that by optimizing the concentration for each of the proteins in combination could increase the potential for axonal outgrowth through the glial scar while decreasing GFAP and CS-56 expression.

The lower pixel intensity of GFAP in the individual treatments, meaning less astrocyte reactivity, may be due to the proteins not activating the signaling pathway involved GFAP expression. This also correlates to the CS-56 pixel intensity. Some studies have used chABC to digest the CSPGs in the inhibitory environment (Bradbury et al. 2002; Zuo et al. 1998; Zuo et al. 2002). The disadvantage of this extrinsic strategy is that it modifies all CSPGs, even those that were deposited during development to maintain axonal tract structure. Digestion of these CSPGs can lead to aberrant axonal outgrowth across tracts, thereby possibly altering connections and functions. The transduction of CA-Cdc42 only affects the levels of CSPGs that comprise the glial scar, while the CSPGs present before the SCI remain intact.

Although the microglia/macrophage response peaks at 2 weeks and decreases after 4 weeks, the ED-1^+ cells were counted at 6 weeks to observe whether a chronic inflammatory response occurred due to our hydrogel/microtubule delivery system. A significant difference was not observed in the number of ED-1^+ cells when the untreated was compared to when agarose was injected alone or in the treatment conditions, BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 (Fig. 4.12). Therefore, the scaffold delivery system does not elicit a long-term inflammatory response and demonstrates potential for future therapeutic use.

In addition to initial injury, secondary injury leads to the increase in the lesion size. A study showed that delivery of anti-tumor growth factor- β (anti-TGF-β) increased

secondary injury, thus increasing the lesion area (King et al. 2004). In this study, the lesion area was measured to observe whether there was a similar occurrence. However, the treated groups, BDNF, CA-Cdc42, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 decreased the lesion area compared to the controls rather than increasing the area of the lesion,. This suggests that BDNF, CA-Cdc42, and CA-Rac1 may have influenced the reactivity and migratory response of the microglia and macrophages, which lead to a decrease in inflammatory cytokine production, thus reducing the astrocytic response. If the reactive astrocytes response has decreased, this in turn affects the GFAP and CSPG production.

Inhibitory regions, identified by the fluorescent intensity of CS-56 were observed proximal to the glial scar in spinal cord and similar in intensity values. When the astrocytes become reactive after injury, these cells deposit CSPGs creating a glial scar. However, it has also been shown that macrophages/microglia are responsible for the deposition of inhibitory CSPGs (Jones et al. 2002). Therefore, the CS-56 intense regions observed proximal to the lesion site are possibly due to the reactive microglia and macrophages. Although a large percentage of axons stopped in the middle of the inhibitory region in the treated conditions, a significant percentage of axons crossed the distal interface of the inhibitory region (Fig. 4.15A). This suggests that the delivered proteins promoted axonal outgrowth through the inhibitory regions. In the study where BDNF conjugated Rhodamine was delivered using the hydrogel/microtubule system. It was observed after 2 weeks that BDNF had diffused 1 to 2 mm proximal to the lesion site, suggesting that the proteins diffused through the tissue to influence axonal outgrowth through the inhibitory regions. Therefore, the proteins diffused to the areas of

the inhibitory region to affect axonal outgrowth. The presence of the CSPG-rich inhibitory regions and the affect of the Rho GTPases and BDNF on axonal outgrowth proximal to the lesion site, suggests that it is imperative to deliver proteins to down-regulate inhibitory molecules not only into the cavity, but also into the proximal area of the lesion site to increase the number axons extending towards the lesion site.

Four weeks post-injury, the anterograde neuronal tracer, BDA, was injected into the motor cortex to trace the axons in the CST towards the lesion site. In the controls, as well as the treated groups, BDA^+ axons did not penetrate into the scaffold-filled cavity. However, axons did penetrate the glial scar in the groups treated with BDNF, CA-Rac1, BDNF/CA-Cdc2, and BDNF/CA-Rac1, where axons had traveled within 200-300 μm to the lesion site (Fig. 4.17), which is within the glial scar as shown in the GFAP and CS-56 intensity graphs (Figs. 4.10 and 4.11). In animals that were injured but were not treated, as well as the animals injected only with agarose, approximately 50% of the axons had retracted 2mm away and the remaining axons stopped 1 mm away from the lesion site (Fig. 4.18). The Rho GTPases, as well as BDNF, influence actin cytoskeleton dynamics. These proteins aid in the polymerization of the actin, which may have allowed axons that retracted in the treated conditions to extend towards the lesion site despite the inhibitory regions. However, it is also possible that the CA-Cdc42, CA-Rac1, and BDNF hindered the depolymerization process of the actin, which causes axonal retraction. Therefore, the axons in the treated groups are close to the lesion site compared to the controls.

Axons from the CST failed to regenerate into the hydrogel scaffold-filled spinal cord cavity. This finding led to the question that perhaps the hydrogel scaffold did not permit axonal penetration. Therefore, spinal cord sections were stained with anti-NF-

160, and it was observed that the cavity in the treated conditions were positive for NF-160 (Fig. 4.19). Although the CST axons did not cross the entire proximal side of the glial scar into the lesion site, axons from other tracts were able to infiltrate the inhibitory glial scar and the scaffold and extend a significant distance in the treated conditions compared to the untreated and agarose controls (Fig. 4.20). Serotonergic (5-HT^{+}) axons in the spinal cord were stained to identify whether these axons were a population of the NF-160^{+} fibers found in the scaffold. Although serotonergic axons were present in the scaffold, these fibers were not a significant population (data not shown). The origin of the NF-160^{+} axons could potentially be fibers from ascending sensory tracts. The ascending sensory tracts are located in the dorsal column of the spinal cord, which were also severed at the time of injury. *In vivo* studies have shown that BDNF helps stimulate axonal outgrowth of sensory fibers (Oudega and Hagg 1999; Zhou and Shine 2003). Due to convergence of pathways between BDNF and the Rho GTPases, CA-Cdc42 and CA-Rac1 could also aid in axonal outgrowth of the sensory fibers.

Individual treatments and combination treatments of CA-Cdc42, CA-Rac1, and BDNF were delivered after SCI. Quantification of GFAP, CS-56, BDA, NF-160 stains did not demonstrate a significant difference between the individual treatments and the combination treatments. However, treatment with CA-Cdc42 demonstrated a reduction in GFAP and CS-56 fluorescent intensity, thereby reducing astrocytes reactivity and CSPG deposition, where as treatment with CA-Rac1 demonstrated a higher percentage of BDA^{+} axonal extension towards the lesion site. This suggests that Cdc42 influences the inflammatory response by affecting the macrophage/microglia and astrocyte responses and Rac1 had more of an affect on neurons and their axonal outgrowth.

In this study, the combinations of BDNF/CA-Cdc42 and BDNF/CA-Rac1 were delivered to the lesion site after SCI. However, the combination of CA-Cdc42/CA-Rac1 or delivery of CA-Cdc2, CA-Rac1, and BDNF in combination was not performed in this study due to cytotoxic effects that were observed in the neurons when CA-Cdc42 and CA-Rac1 were transduced together (Jain et al. 2004). The BDNF diffusion study in vitro demonstrates that the microtubules release protein for at least 2 weeks. The delivery of BDNF, CA-Cdc42, and CA-Rac1 individually and in combination encouraged axons to extend through CSPG inhibitory regions proximal to the lesion site. These proteins delivered individually also reduced the inflammatory response. Although axonal outgrowth was not observed in the scaffold-filled lesion site in the treated conditions, axons extended a significant distance compared to the untreated and agarose treated spinal cords suggesting that the proteins were encouraging axons through the inhibitory region. These results suggest that the proteins delivered in this study could possibly promote axonal outgrowth into the lesion site if the concentration of delivered CA-Cdc42, CA-Rac1, and BDNF were optimized.

4.5. ACKNOWLEDGMENTS

This work was supported by a grant from National Institutes of Health R01 NS43486 (RVB). Support from GTEC (EEC-9731643), an NSF Engineering Research Center based at Georgia Tech/Emory is also acknowledged for use of its core facilities.

4.6. REFERENCES

Balgude AP, Yu X, Szymanski A, Bellamkonda RV. 2001. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. Biomaterials 22(10):1077-1084.

Bellamkonda R, Ranieri JP, Aebischer P. 1995a. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension in vitro. J Neurosci Res 41(4):501-509.

Bellamkonda R, Ranieri JP, Bouche N, Aebischer P. 1995b. Hydrogel-based three-dimensional matrix for neural cells. J Biomed Mater Res 29(5):663-671.

Borkenhagen M, Clemence JF, Sigrist H, Aebischer P. 1998. Three-dimensional extracellular matrix engineering in the nervous system. J Biomed Mater Res 40(3):392-400.

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(6881):636-640.

Chan CC, Wong AK, Liu J, Steeves JD, Tetzlaff W. 2007. ROCK inhibition with Y27632 activates astrocytes and increases their expression of neurite growth-inhibitory chondroitin sulfate proteoglycans. Glia 55(4):369-384.

Chen TJ, Gehler S, Shaw AE, Bamburg JR, Letourneau PC. 2006. Cdc42 participates in the regulation of ADF/cofilin and retinal growth cone filopodia by brain derived neurotrophic factor. J Neurobiol 66(2):103-114.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. 2002. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22(15):6570-6577.

Dillon GP, Yu X, Bellamkonda RV. 2000. The polarity and magnitude of ambient charge influences three-dimensional neurite extension from DRGs. J Biomed Mater Res 51(3):510-519.

Dillon GP, Yu X, Sridharan A, Ranieri JP, Bellamkonda RV. 1998. The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold. J Biomater Sci Polym Ed 9(10):1049-1069.

Dubey N, Letourneau PC, Tranquillo RT. 1999. Guided neurite elongation and schwann cell invasion into magnetically aligned collagen in simulated peripheral nerve regeneration. Exp Neurol 158(2):338-350.

Evans GR. 2001. Peripheral nerve injury: a review and approach to tissue engineered constructs. Anat Rec 263(4):396-404.

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23(4):1416-1423.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509-514.

Holtje M, Hoffmann A, Hofmann F, Mucke C, Grosse G, Van Rooijen N, Kettenmann H, Just I, Ahnert-Hilger G. 2005. Role of Rho GTPase in astrocyte morphology and migratory response during in vitro wound healing. J Neurochem 95(5):1237-1248.

Houweling DA, Lankhorst AJ, Gispen WH, Bar PR, Joosten EA. 1998. Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons

in the adult rat spinal cord and promotes partial functional recovery. Exp Neurol 153(1):49-59.

Jain A, Brady-Kalnay SM, Bellamkonda RV. 2004. Modulation of Rho GTPase activity alleviates chondroitin sulfate proteoglycan-dependent inhibition of neurite extension. J Neurosci Res 77(2):299-307.

Jain A, Kim YT, McKeon RJ, Bellamkonda RV. 2006. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. Biomaterials 27(3):497-504.

Jin Z, Strittmatter SM. 1997. Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17(16):6256-6263.

Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J Neurosci 22(7):2792-2803.

Kataoka K, Suzuki Y, Kitada M, Hashimoto T, Chou H, Bai H, Ohta M, Wu S, Suzuki K, Ide C. 2004. Alginate enhances elongation of early regenerating axons in spinal cord of young rats. Tissue Eng 10(3-4):493-504.

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(1):173-183.

Krewson CE, Klarman ML, Saltzman WM. 1995. Distribution of nerve growth factor following direct delivery to brain interstitium. Brain Res 680(1-2):196-206.

Labrador RO, Buti M, Navarro X. 1998. Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair. Exp Neurol 149(1):243-252.

Meilander NJ, Pasumarthy MK, Kowalczyk TH, Cooper MJ, Bellamkonda RV. 2003. Sustained release of plasmid DNA using lipid microtubules and agarose hydrogel. J Control Release 88(2):321-331.

Meilander NJ, Yu X, Ziats NP, Bellamkonda RV. 2001. Lipid-based microtubular drug delivery vehicles. J Control Release 71(1):141-152.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22(3):319-330.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Novikova LN, Novikov LN, Kellerth JO. 2000. Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. Eur J Neurosci 12(2):776-780.

Oudega M, Hagg T. 1999. Neurotrophins promote regeneration of sensory axons in the adult rat spinal cord. Brain Res 818(2):431-438.

Sayer FT, Oudega M, Hagg T. 2002. Neurotrophins reduce degeneration of injured ascending sensory and corticospinal motor axons in adult rat spinal cord. Exp Neurol 175(1):282-296.

Schmidt CE, Leach JB. 2003. Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng 5:293-347.

Schnur JM. 1993. Lipid Tubules: A Paradigm for Molecularly Engineered Scaffolds. Science 262:1669-1676.

Shimizu TaJ, G. 2002. Molecule-Up Fabrication and Manipulation of Lipid Nanotubes. International Journal of Nanoscience 1(5&6):465-469.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L. 2002. Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277(36):32820-32829.

Woerly S, Doan VD, Evans-Martin F, Paramore CG, Peduzzi JD. 2001. Spinal cord reconstruction using NeuroGel implants and functional recovery after chronic injury. J Neurosci Res 66(6):1187-1197.

Woerly S, Doan VD, Sosa N, de Vellis J, Espinosa-Jeffrey A. 2004. Prevention of gliotic scar formation by NeuroGel allows partial endogenous repair of transected cat spinal cord. J Neurosci Res 75(2):262-272.

Yu X, Bellamkonda RV. 2003. Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Eng 9(3):421-430.

Yuan XB, Jin M, Xu X, Song YQ, Wu CP, Poo MM, Duan S. 2003. Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat Cell Biol 5(1):38-45.

Zhou L, Shine HD. 2003. Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. J Neurosci Res 74(2):221-226.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D. 1998. Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Exp Neurol 154(2):654-662.

Zuo J, Neubauer D, Graham J, Krekoski CA, Ferguson TA, Muir D. 2002. Regeneration of axons after nerve transection repair is enhanced by degradation of chondroitin sulfate proteoglycan. Exp Neurol 176(1):221-228.

CHAPTER 5

CONCLUSIONS

The global objective of this study was to investigate whether the Rho GTPases could mask the effect of the inhibitory molecules, particularly CSPGs, in order to encourage axonal outgrowth. To accomplish this objective, the study was divided into two parts, one which was conducted *in vitro* and the other *in vivo*. The study performed *in vitro* was to determine whether modulating Rho GTPases, Cdc42 and Rac1, in neurons could influence axonal outgrowth into CSPG-rich regions. To conduct a study *in vivo* the following sub-goals were set: (1) to develop a 3-dimensional (3D) scaffold that conformally filled the spinal cord cavity and allow for the axons to infiltrate the hydrogel; (2) to utilize a delivery vehicle that locally delivered the proteins within the lesion site into the surrounding tissue, permitting the transduction of the proteins into the neurons; and (3) to deliver Rho GTPases, which had demonstrated *in vitro* could affect neurite extension through inhibitory regions after transduction, and BDNF to observe whether axonal outgrowth occurred through CSPG-rich regions.

In the *in vitro* study the Rho GTPases were modulated to alleviate CSPG-dependent inhibition on neurite extension. Studies had previously shown in similar *in vitro* models that the inhibition of RhoA or its downstream effector by C3 or Y27632, respectively, had prevented growth cone collapse and increased neuronal extension (Dubreuil et al. 2003; Monnier et al. 2003; Winton et al. 2002). Although RhoA can be inhibited to prevent growth cone collapse, this does not signify an increase in the

activation of filopodia and lamellopodia on growth cones to read the environmental cues and promote neurite extension through the inhibitory regions. Therefore, we modulated Cdc42 and Rac1, which directly induced filopodial and lamellopodial extensions, in the mutant derivative forms, as well as RhoA and C3 to determine if there was an increase in neurite crossing through inhibitory CSPG regions.

The molecular weight of the Rho GTPases does not allow the proteins to cross the cellular membrane without the aid of a delivery vehicle. Chariot, a commercially available peptide, was complexed to transduce the proteins across the membrane. A significantly higher percent ratio of neurites crossing the inhibitory regions was observed when CA-Rac1 was complexed to Chariot versus the media control or when CA-Rac1 was added to the media alone. Our study demonstrated the necessity and success of the peptide delivery system to effectively transduce the Rho GTPases across the membrane in order for CA-Rac1 and CA-Cdc42 to affect the signaling transduction pathway involved in actin cytoskeleton dynamics.

CA-Cdc42 was transduced into neurons at various concentrations: 1 μg/ml, 2 μg/ml, 3 μg/ml, 4 μg/ml, 6 μg/ml and 8 μg/ml. Although the differences amongst the concentrations were very small, the cellular response was strikingly different. The percent ratios for the concentrations between 1 μg/ml and 3 μg/ml were not significantly different from the negative controls, while the cytotoxic effects were observed in the neurons when 6 μg/ml and 8 μg/ml of protein were delivered, either due to too high protein concentration or the Chariot concentrations levels. However, significantly higher percent ratio of neurite crossing was seen for 4 μg/ml, the middle concentration. This

demonstrates that activation versus inactivation of a signal transduction pathway can occur due to slight differences in intracellular protein concentrations.

From the *in vitro* study, we concluded that transduction of CA-Cdc42 and CA-Rac1, endogenous proteins, promoted axonal outgrowth through inhibitory regions. Delivery of C3, a bacterial toxin, demonstrated similar results. Athough C3 may hinder growth cone collapse by inhibiting the Rho signaling pathway, this does not translate to the activation of filopodia and lamellopodia such that the neurites can overcome CSPG-dependent inhibition. Therefore, when developing the experimental design *in vivo,* only CA-Cdc42 and CA-Rac1 were transduced into neurons after injury, along with BDNF.

A delivery system, which included a hydrogel scaffold and delivery vehicle, was developed to slowly release CA-Cdc42, CA-Rac1, and BDNF locally. Agarose gels of different gelling temperatures and gel strengths were investigated to develop an *in situ* gelling hydrogel and to promote axonal infiltration. Based on the rheology data and DRG cultures, it was determined that SeaPrep® agarose is the most conducive as a scaffold for delivery and axonal infiltration. The gelling temperature for this agarose was 11°C. An *in situ* gel cooling system was developed so that the agarose could gel within the created cavity. An agarose scaffold that gelled *in situ* rather than pre-fabricated is more desirable because it takes the shape of the defect, thereby minimizing the physical space between the spinal cord tissue and the scaffold. Although an approximately same size cavity was made for the animals in all of the conditions, slight differences would preclude the conformal filling of the cavity by a pre-fabricated scaffold. An *in situ* gelling scaffold also has clinical relevance. Patients suffering from SCI have different size lesions and a pre-fabricated scaffold would demand time that may not be available to

resize the scaffold, where as *in situ* gelling scaffold could be used immediately irregardless of the cavity size.

The agarose scaffold contained lipid microtubules that slowly and locally delivered the proteins. The lipid microtubules were chosen as the delivery vehicle because it had been shown previously that the microtubules could deliver the proteins for several weeks. The *in vitro* release assay demonstrated that the microtubules slowly released BDNF for at least 2 weeks. This time period is biologically crucial because the glial scar is forming and the severed axons are retracting. Therefore, the release of the CA-Cdc42, CA-Rac1, and BDNF from the microtubules during the first two weeks after injury into the surrounding tissue allows for accessibility to the neurons and allows the retracted axons to stall or extend forward. The quantification of ED-1^{+} stain for macrophages/reactive microglia at 6 weeks exhibited that the hydrogel/ microtubule delivery system did not elicit a chronic inflammatory response when compared to the untreated controls. This signifies that the microtubules did not provoke the inflammatory response. It also does not seem that the microtubules affect the bioactivity of the proteins. In comparison with other delivery vehicles, osmotic pump, microparticles, and Gelfoam, using microtubules is more advantageous. The insertion of the catheter from the osmotic pump induces another injury that needs to be treated and microspheres have been phagocytosed by macrophages. As mentioned above, the microtubules have shown to release for at least 2 weeks *in vitro*; however, the release of proteins from Gelfoam cannot be controlled and releases the proteins in large amounts over a short period of time.

The conclusions drawn from the *in vitro* studies encouraged the investigation of Cdc42 and Rac1 to be delivered *in vivo*. BDNF was also chosen because it demonstrated neuroprotective behavior, as well as its effect on actin cytoskeleton. CA-Cdc42 and CA-Rac1 were delivered in combination with BDNF to determine whether synergistic effects occurred. However, CA-Cdc42 and CA-Rac1 were not delivered in combination due to the cytotoxic effects observed in the *in vitro* study. The effects of delivery of CA-Cdc42, CA-Rac1, and BDNF were analyzed by staining for the astrocytic response using the antibody against GFAP, CSPG deposition with anti-CS-56, and axons after injection of BDA and NF-160. The quantification of GFAP pixel intensity demonstrated that when the proteins were delivered individually, the intensity of reactive astrocytes was significantly lower than the controls, however, when the proteins were delivered in combination, the fluorescent intensity was similar to the untreated and agarose controls. This could be due to the amount of protein delivered causing the astrocytes to be more migratory, thereby increasing GFAP expression. However, when the proteins were delivered individually, the pathway was not activated due the protein levels. It is also possible that an alternative signaling pathway was activated causing a reduction in astrocyte activity. A similar trend was observed with the CS-56 staining near the lesion site. The GFAP intensity was lower signifying less reactive astrocytes, thereby reducing secretion of CSPGs.

After injury, axonal retraction occurs. In the case of injured untreated animals, axonal retraction can occur up to 4 mm rostral to the lesion site. In the treated groups, a higher percentage of axons were shown between the beginning of the lesion site and 1

mm proximal, especially in the CA-Rac1, and BDNF/CA-Cdc42 and BDNF/CA-Rac1 treatment groups.

CS-56 stained inhibitory regions were seen proximal to the lesion site. Axonal outgrowth through these regions was quantified similar to the *in vitro* study quantifying the percent ratio of neurite crossing the CSPG-rich regions. The results demonstrated that the treatment groups had a significantly higher number of axons that crossed the distal interface of the inhibitory region. It can also be seen from the data showing the average axonal retraction from the beginning of the lesion site that the groups treated with BDNF, CA-Rac1, BDNF/CA-Cdc42, and BDNF/CA-Rac1 had axons that infiltrated the glial scar; however, these axons stopped before the lesion site.

The McKerracher and Strittmatter Laboratories have delivered C3 after SCI to determine whether CST axons would regenerate. The results from the two labs were contradictory. McKerracher's group stated that sprouting was observed into the lesion site after treatment with C3 (Dergham et al. 2002), whereas Strittmatter's groups stated that C3 did not promote any sprouting or long-distance regeneration (Fournier et al. 2003). The delivery of C3 affects the Rho signaling pathway. In order to affect the growth cone cytoskeleton dynamics, it requires cross-talk between Rho and Cdc42 and Rac. Studies on delivery of Cdc42 and Rac1 *in vivo* after SCI have not been reported. Our studies report that it is more beneficial to deliver Cdc42 and Rac1 to promote axonal outgrowth. We have observed beneficial effects of delivering CA-Cdc42 and CA-Rac1 on axonal growth. However, we have not been able to extrapolate whether the proteins affected the signaling pathway for actin polymerization or depolymerization. It would be

a worthwhile study in the future to perform immunoblots for proteins in the two signaling pathways to determine which pathway is activated more.

The other approaches, discussed in Chapter 2, observed axonal regrowth and/or functional recovery. It is difficult to make a comparison between those studies and ours due to difference in parameters, such as the delivered dosage, time of protein delivery, and duration of the study. The benefits of our strategy are the local delivery of proteins, lack of an immune response to the proteins, and an *in situ* conformally filling scaffold. Strategies that transplant cells or use viral vectors raise concern about the host immune response, which raises issues of clinical relevance. The advantages of our lipid microtubule delivery system compared to other delivery systems were mentioned above. Although axonal outgrowth was not observed in the lesion site, the delivery of Cdc42 and Rac1 shows potential with their effect on the astroglial scar and axonal outgrowth. It has become evident that a multi-factorial approach, a combination of treatments, is imperative for regeneration of the spinal cord. The studies presented in this thesis have demonstrated that Cdc42 and Rac1 should be pursued and considered as players in the strategy to promote axonal regeneration and achieve full functional recovery.

REFERENCES

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. 2002. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22(15):6570-6577.

Dubreuil CI, Winton MJ, McKerracher L. 2003. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. J Cell Biol 162(2):233-243.

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23(4):1416-1423.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22(3):319-330.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L. 2002. Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277(36):32820-32829.

CHAPTER 6

FUTURE PERSPECTIVES

Axonal regeneration into the distal spinal cord, reforming proper connections, leading to full functional recovery, has yet to be accomplished. Our study investigated the potential of Cdc42, Rac1, and BDNF to encourage axons to extend through inhibitory regions. However, the ultimate goal is to study whether these proteins can promote axonal outgrowth through the scaffold-filled cavity into the distal portion of the spinal cord. This chapter will discuss further aspects that can be studied to determine whether this can be achieved.

6.1. OPTIMIZATION OF DOSAGE

In the *in vivo* study presented in Chapter 4, one concentration for each of the proteins was delivered to observe the effect on the axons, particularly in the CST, as well as the inflammatory response. In the *in vitro* study presented in Chapter 3, it was observed that of the five different dosages delivered to the DRGs, it was the 4 μg/mL that elicited the greatest response. Therefore, delivering different concentrations of CA-Cdc42, CA-Rac1, and BDNF to determine the optimal dosage may result in axonal outgrowth through the scaffold into the distal portion of the spinal cord.

Combinations of BDNF/CA-Cdc42 and BDNF/CA-Rac1 were delivered into the spinal cord lesion (Chapter 4). The optimal dosage delivered for each of the proteins individually will not necessarily translate to the best possible dosage delivered in

combination. Therefore, it is necessary to perform studies that optimize the concentration of each protein when delivered in combination. It was observed that when BDNF was delivered in combination with Cdc42 or Rac1, there was a greater inflammatory response in terms of the reactive astrocytes and deposition of CSPGs. If the concentrations of the two proteins are altered, then the responses maybe greater or lesser than what was observed in our *in vivo* study.

6.2. OPTIMIZATION OF DELIVERY VEHICLE

Lipid microtubules were used to deliver the proteins in our *in vivo* study. An *in vitro* experiment was conducted, demonstrating that the microtubules release the proteins for at least 2 weeks. If desired, longer microtubules could be fabricated to increase the duration of release of the proteins. Nanoparticles can also be used to deliver the proteins. Although nanoparticles risk being phagocytosed, if the copolymer, such as PLGA, is used at different ratios of the two monomers, it allows for control of the degradation of the delivery vehicle, thereby controlling the period of time the protein is released. It would be interesting to observe the effect on axonal outgrowth that delivering the proteins had at various time points post-injury. If the proteins were delivered during the 3rd to 5th weeks post-injury, as well as immediately after injury, it may encourage axons to traverse the scaffold and penetrate the distal spinal cord.

6.3. MODIFICATION OF SCAFFOLD

We used agarose as the scaffold in the *in vivo* study. It is possible to crosslink proteins in order to modify the hydrogels (reviewed in Chapter 2). Laminin has been

shown to promote axonal outgrowth. Therefore, crosslinking laminin to the hydrogel scaffold may aid in axonal infiltration into the scaffold.

6.4. DURATION OF *IN VIVO* STUDY

The duration of the *in vivo* study conducted for this thesis was 6 weeks. If the study had been terminated later, such as at 8 or 10 weeks, axonal outgrowth might have been observed closer to the lesion, or even into the scaffold-filled cavity. Also, behavioral analysis at these timepoints might have shown functional recovery.

6.5. SHORT-TERM STUDY OF INFLAMMATORY RESPONSE

In the *in vivo* study, no significant difference was observed in the ED-1^+ cells between the treatment groups and the controls at 6 weeks. Our study showed that the Rho GTPases and BDNF affected the reactive astrocyte response and CSPG deposition. The number of macrophages and reactive microglia peaks at 2 weeks and begins to fall by 4 weeks. The shorter time studies should be performed *in vivo* to determine whether activation of microglia and the migration of macrophages were affected. Because these cells secrete cytokines that activate astrocytes and inhibitory molecules, additional studies would help explain how the glial scar is being altered.

6.6. MULTI-FACTORIAL APPROACH

A multi-factorial approach, rather than a unilateral one, will be necessary to restore complete functional recovery. In our *in vivo* study, we observed that CSPG inhibitory regions, possibly formed after CSPG secretion by macrophages/reactive

microglia proximal to the lesion site, inhibited a high percentage of axons prior to the glial scar. Therefore, for future studies it would be important in the experimental design to include delivery of an anti-inflammatory agent approximately 1-2 mm proximal to the lesion site, which decreased macrophages/reactive microglia, thus preventing the deposition of CSPGs.

There are two main strategies to promote axonal outgrowth: intrinsic and extrinsic. These studies investigated the use of the intrinsic strategy, by utilizing Rho GTPases and their role in actin cytoskeleton dynamics. However, it would be interesting to determine whether a study that combines both strategies would have a greater effect on the promotion of axonal outgrowth. An example would be to deliver either CA-Cdc42 or CA-Rac1 in combination with siRNA, which hinders the production of specific CSPGs. CA-Cdc42 and CA-Rac1 should encourage the growth cones on the retracted axons to read the environmental cues and extend further while the delivery of the siRNA against CSPGs would prevent the production of the protein by the reactive astrocytes, thus altering the composition of the glial scar, making it less inhibitory and more conducive for axonal outgrowth. The Strittmatter group demonstrated that genetically modified astroyctes secreted chABC, which could also be used to remove the CSPGs from the glial scar, as well as provide a substrate for axonal outgrowth (Chapter 2). Other strategies mentioned could also be used in combination with the Rho GTPases to obtain axonal regeneration.

REFERENCES

Abdellatif AA, Pelt JL, Benton RL, Howard RM, Tsoulfas P, Ping P, Xu XM, Whittemore SR. 2006. Gene delivery to the spinal cord: comparison between lentiviral, adenoviral, and retroviral vector delivery systems. J Neurosci Res 84(3):553-567.

Aguayo AJ, David S, Bray GM. 1981. Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. The Journal of experimental biology 95:231-240.

Azzouz M, Kingsman SM, Mazarakis ND. 2004. Lentiviral vectors for treating and modeling human CNS disorders. The journal of gene medicine 6(9):951-962.

Balgude AP, Yu X, Szymanski A, Bellamkonda RV. 2001. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. Biomaterials 22(10):1077-1084.

Bandtlow CE. 2003. Regeneration in the central nervous system. Exp Gerontol 38(1-2):79-86.

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(6):1375-1381.

Bellamkonda R, Ranieri JP, Aebischer P. 1995a. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension in vitro. J Neurosci Res 41(4):501-509.

Bellamkonda R, Ranieri JP, Bouche N, Aebischer P. 1995b. Hydrogel-based three-dimensional matrix for neural cells. J Biomed Mater Res 29(5):663-671.

Biran R, Noble MD, Tresco PA. 2003. Directed nerve outgrowth is enhanced by engineered glial substrates. Exp Neurol 184(1):141-152.

Blits B, Bunge MB. 2006. Direct gene therapy for repair of the spinal cord. J Neurotrauma 23(3-4):508-520.

Blits B, Oudega M, Boer GJ, Bartlett Bunge M, Verhaagen J. 2003. Adeno-associated viral vector-mediated neurotrophin gene transfer in the injured adult rat spinal cord improves hind-limb function. Neuroscience 118(1):271-281.

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W. 2003. Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci 22(3):405-416.

Borkenhagen M, Clemence JF, Sigrist H, Aebischer P. 1998. Three-dimensional extracellular matrix engineering in the nervous system. J Biomed Mater Res 40(3):392-400.

Bradbury EJ, Khemani S, Von R, King, Priestley JV, McMahon SB. 1999. NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. Eur J Neurosci 11(11):3873-3883.

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(6881):636-640.

Brosamle C, Huber AB, Fiedler M, Skerra A, Schwab ME. 2000. Regeneration of lesioned corticospinal tract fibers in the adult rat induced by a recombinant, humanized IN-1 antibody fragment. J Neurosci 20(21):8061-8068.

Cafferty WB, Yang SH, Duffy PJ, Li S, Strittmatter SM. 2007. Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans. J Neurosci 27(9):2176-2185.

Camarata PJ, Suryanarayanan R, Turner DA, Parker RG, Ebner TJ. 1992. Sustained release of nerve growth factor from biodegradable polymer microspheres. Neurosurgery 30(3):313-319.

Cao X, Schoichet MS. 1999. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. Biomaterials 20(4):329-339.

Ceballos D, Navarro X, Dubey N, Wendelschafer-Crabb G, Kennedy WR, Tranquillo RT. 1999. Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration. Exp Neurol 158(2):290-300.

Chan CC, Wong AK, Liu J, Steeves JD, Tetzlaff W. 2007. ROCK inhibition with Y27632 activates astrocytes and increases their expression of neurite growth-inhibitory chondroitin sulfate proteoglycans. Glia 55(4):369-384.

Chen TJ, Gehler S, Shaw AE, Bamburg JR, Letourneau PC. 2006. Cdc42 participates in the regulation of ADF/cofilin and retinal growth cone filopodia by brain derived neurotrophic factor. J Neurobiol 66(2):103-114.

Chen ZJ, Ughrin Y, Levine JM. 2002. Inhibition of axon growth by oligodendrocyte precursor cells. Mol Cell Neurosci 20(1):125-139.

Condic ML, Lemons ML. 2002. Extracellular matrix in spinal cord regeneration: getting beyond attraction and inhibition. Neuroreport 13(3):A37-48.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81(7):1137-1146.

David S, Lacroix S. 2003. Molecular Approaches to Spinal Cord Repair. Annu Rev Neurosci.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. 2002. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22(15):6570-6577.

Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F. 2004. Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. Biochemistry 43(6):1449-1457.

Deumens R, Koopmans GC, Den Bakker CG, Maquet V, Blacher S, Honig WM, Jerome R, Pirard JP, Steinbusch HW, Joosten EA. 2004. Alignment of glial cells stimulates directional neurite growth of CNS neurons in vitro. Neuroscience 125(3):591-604.

Dillon GP, Yu X, Bellamkonda RV. 2000. The polarity and magnitude of ambient charge influences three-dimensional neurite extension from DRGs. J Biomed Mater Res 51(3):510-519.

Dillon GP, Yu X, Sridharan A, Ranieri JP, Bellamkonda RV. 1998. The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold. J Biomater Sci Polym Ed 9(10):1049-1069.

Doetsch F. 2003. The glial identity of neural stem cells. Nat Neurosci 6(11):1127-1134.

Dubey N, Letourneau PC, Tranquillo RT. 1999. Guided neurite elongation and schwann cell invasion into magnetically aligned collagen in simulated peripheral nerve regeneration. Exp Neurol 158(2):338-350.

Dubreuil CI, Winton MJ, McKerracher L. 2003. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. J Cell Biol 162(2):233-243.

Evans GR. 2001. Peripheral nerve injury: a review and approach to tissue engineered constructs. Anat Rec 263(4):396-404.

Fawcett JW, Asher RA. 1999. The glial scar and central nervous system repair. Brain Res Bull 49(6):377-391.

Filbin MT. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4(9):703-713.

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23(4):1416-1423.

Fricker-Gates RA. 2006. Radial glia: a changing role in the central nervous system. Neuroreport 17(11):1081-1084.

GrandPre T, Li S, Strittmatter SM. 2002. Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417(6888):547-551.

Green I, Christison R, Voyce CJ, Bundell KR, Lindsay MA. 2003. Protein transduction domains: are they delivering? Trends Pharmacol Sci 24(5):213-215.

Gros E, Deshayes S, Morris MC, Aldrian-Herrada G, Depollier J, Heitz F, Divita G. 2006. A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction. Biochimica et biophysica acta 1758(3):384-393.

Grumet M, Flaccus A, Margolis RU. 1993. Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules. J Cell Biol 120(3):815-824.

Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P. 1992. Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. J Neurosci 12(9):3310-3320.

Guo JS, Zeng YS, Li HB, Huang WL, Liu RY, Li XB, Ding Y, Wu LZ, Cai DZ. 2007. Cotransplant of neural stem cells and NT-3 gene modified Schwann cells promote the recovery of transected spinal cord injury. Spinal Cord 45(1):15-24.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509-514.

Hall A, Nobes CD. 2000. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci 355(1399):965-970.

Hennigan A, O'Callaghan RM, Kelly AM. 2007. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochemical Society transactions 35(Pt 2):424-427.

Hiebert GW, Khodarahmi K, McGraw J, Steeves JD, Tetzlaff W. 2002. Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. J Neurosci Res 69(2):160-168.

Hoke A, Silver J. 1996. Proteoglycans and other repulsive molecules in glial boundaries during development and regeneration of the nervous system. Prog Brain Res 108:149-163.

Holtje M, Hoffmann A, Hofmann F, Mucke C, Grosse G, Van Rooijen N, Kettenmann H, Just I, Ahnert-Hilger G. 2005. Role of Rho GTPase in astrocyte morphology and migratory response during in vitro wound healing. J Neurochem 95(5):1237-1248.

Houweling DA, Lankhorst AJ, Gispen WH, Bar PR, Joosten EA. 1998. Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. Exp Neurol 153(1):49-59.

Jain A, Brady-Kalnay SM, Bellamkonda RV. 2004. Modulation of Rho GTPase activity alleviates chondroitin sulfate proteoglycan-dependent inhibition of neurite extension. J Neurosci Res 77(2):299-307.

Jain A, Kim YT, McKeon RJ, Bellamkonda RV. 2006. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. Biomaterials 27(3):497-504.

Jin Z, Strittmatter SM. 1997. Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17(16):6256-6263.

Joliot A, Prochiantz A. 2004. Transduction peptides: from technology to physiology. Nat Cell Biol 6(3):189-196.

Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J Neurosci 22(7):2792-2803.

Kataoka K, Suzuki Y, Kitada M, Hashimoto T, Chou H, Bai H, Ohta M, Wu S, Suzuki K, Ide C. 2004. Alginate enhances elongation of early regenerating axons in spinal cord of young rats. Tissue Eng 10(3-4):493-504.

Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O. 2005. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25(19):4694-4705.

Khan T, Dauzvardis M, Sayers S. 1991. Carbon filament implants promote axonal growth across the transected rat spinal cord. Brain Res 541(1):139-145.

Kim DH, Jahng TA. 2004. Continuous brain-derived neurotrophic factor (BDNF) infusion after methylprednisolone treatment in severe spinal cord injury. J Korean Med Sci 19(1):113-122.

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(1):173-183.

Kjoller L, Hall A. 1999. Signaling to Rho GTPases. Exp Cell Res 253(1):166-179.

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(6):1566-1569.

Kozma R, Ahmed S, Best A, Lim L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15(4):1942-1952.

Krewson CE, Klarman ML, Saltzman WM. 1995. Distribution of nerve growth factor following direct delivery to brain interstitium. Brain Res 680(1-2):196-206.

Kuhn TB, Meberg PJ, Brown MD, Bernstein BW, Minamide LS, Jensen JR, Okada K, Soda EA, Bamburg JR. 2000. Regulating actin dynamics in neuronal growth cones by ADF/cofilin and rho family GTPases. J Neurobiol 44(2):126-144.

Kwon BK, Oxland TR, Tetzlaff W. 2002. Animal models used in spinal cord regeneration research. Spine 27(14):1504-1510.

Labrador RO, Buti M, Navarro X. 1998. Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair. Exp Neurol 149(1):243-252.

Lakatos A, Barnett SC, Franklin RJ. 2003. Olfactory ensheathing cells induce less host astrocyte response and chondroitin sulphate proteoglycan expression than Schwann cells following transplantation into adult CNS white matter. Exp Neurol 184(1):237-246.

Lee AC, Yu VM, Lowe JB, 3rd, Brenner MJ, Hunter DA, Mackinnon SE, Sakiyama-Elbert SE. 2003. Controlled release of nerve growth factor enhances sciatic nerve regeneration. Exp Neurol 184(1):295-303.

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L. 1999. Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19(17):7537-7547.

Levi-Montalcini R. 1987. The nerve growth factor 35 years later. Science 237(4819):1154-1162.

Li S, Strittmatter SM. 2003. Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. J Neurosci 23(10):4219-4227.

Liu LS, Khan T, Sayers ST, Dauzvardis MF, Trausch CL. 1995. Electrophysiological improvement after co-implantation of carbon filaments and fetal tissue in the contused rat spinal cord. Neurosci Lett 200(3):199-202.

Luo L. 2000. Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci 1(3):173-180.

Luo Y, Shoichet MS. 2004. A photolabile hydrogel for guided three-dimensional cell growth and migration. Nat Mater 3(4):249-253.

Mattson MP, Taylor-Hunter A, Kater SB. 1988. Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. J Neurosci 8(5):1704-1711.

McDonald JW, Becker D, Holekamp TF, Howard M, Liu S, Lu A, Lu J, Platik MM, Qu Y, Stewart T, Vadivelu S. 2004. Repair of the injured spinal cord and the potential of embryonic stem cell transplantation. J Neurotrauma 21(4):383-393.

McKeon RJ, Hoke A, Silver J. 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136(1):32-43.

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(4):805-811.

Meilander NJ, Pasumarthy MK, Kowalczyk TH, Cooper MJ, Bellamkonda RV. 2003. Sustained release of plasmid DNA using lipid microtubules and agarose hydrogel. J Control Release 88(2):321-331.

Meilander NJ, Yu X, Ziats NP, Bellamkonda RV. 2001. Lipid-based microtubular drug delivery vehicles. J Control Release 71(1):141-152.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. 2003. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22(3):319-330.

Morgenstern DA, Asher RA, Fawcett JW. 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 137:313-332.

Morris MC, Depollier J, Mery J, Heitz F, Divita G. 2001. A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19(12):1173-1176.

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(3):757-767.

Myckatyn TM, Mackinnon SE, McDonald JW. 2004. Stem cell transplantation and other novel techniques for promoting recovery from spinal cord injury. Transplant immunology 12(3-4):343-358.

Ngo TT, Waggoner PJ, Romero AA, Nelson KD, Eberhart RC, Smith GM. 2003. Poly(L-Lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions. J Neurosci Res 72(2):227-238.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE. 2002. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22(23):10368-10376.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Novikov LN, Novikova LN, Mosahebi A, Wiberg M, Terenghi G, Kellerth JO. 2002. A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury. Biomaterials 23(16):3369-3376.

Novikova LN, Novikov LN, Kellerth JO. 2000. Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. Eur J Neurosci 12(2):776-780.

Novikova LN, Novikov LN, Kellerth JO. 2002. Differential effects of neurotrophins on neuronal survival and axonal regeneration after spinal cord injury in adult rats. J Comp Neurol 452(3):255-263.

Oudega M, Hagg T. 1999. Neurotrophins promote regeneration of sensory axons in the adult rat spinal cord. Brain Res 818(2):431-438.

Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A. 1990. Microinjection of recombinant p21rho induces rapid changes in cell morphology. J Cell Biol 111(3):1001-1007.

Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB. 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10(6):610-616.

Prang P, Muller R, Eljaouhari A, Heckmann K, Kunz W, Weber T, Faber C, Vroemen M, Bogdahn U, Weidner N. 2006. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 27(19):3560-3569.

Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT. 2002. Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34(6):895-903.

Rangappa N, Romero A, Nelson KD, Eberhart RC, Smith GM. 2000. Laminin-coated poly(L-lactide) filaments induce robust neurite growth while providing directional orientation. J Biomed Mater Res 51(4):625-634.

Robbins PD, Tahara H, Ghivizzani SC. 1998. Viral vectors for gene therapy. Trends in biotechnology 16(1):35-40.

Sakiyama-Elbert SE, Hubbell JA. 2000a. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Control Release 69(1):149-158.

Sakiyama-Elbert SE, Hubbell JA. 2000b. Development of fibrin derivatives for controlled release of heparin-binding growth factors. J Control Release 65(3):389-402.

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):1-11.

Santos-Benito FF, Ramon-Cueto A. 2003. Olfactory ensheathing glia transplantation: a therapy to promote repair in the mammalian central nervous system. Anat Rec 271B(1):77-85.

Sayer FT, Oudega M, Hagg T. 2002. Neurotrophins reduce degeneration of injured ascending sensory and corticospinal motor axons in adult rat spinal cord. Exp Neurol 175(1):282-296.

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.

Schmidt CE, Leach JB. 2003. Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng 5:293-347.

Schnur JM. 1993. Lipid Tubules: A Paradigm for Molecularly Engineered Scaffolds. Science 262:1669-1676.

Schweigreiter R, Bandtlow CE. 2006. Nogo in the injured spinal cord. J Neurotrauma 23(3-4):384-396.

Schweigreiter R, Walmsley AR, Niederost B, Zimmermann DR, Oertle T, Casademunt E, Frentzel S, Dechant G, Mir A, Bandtlow CE. 2004. Versican V2 and the central inhibitory domain of Nogo-A inhibit neurite growth via p75NTR/NgR-independent pathways that converge at RhoA. Mol Cell Neurosci 27(2):163-174.

Sci-info-pages.com. March 2002. Spinal Cord Injury Facts & Statistics. http://wwwsci-info-pagescom/factshtml. May 2007

Shaw D, Shoichet MS. 2003. Toward spinal cord injury repair strategies: peptide surface modification of expanded poly(tetrafluoroethylene) fibers for guided neurite outgrowth in vitro. J Craniofac Surg 14(3):308-316.

Shimizu TaJ, G. 2002. Molecule-Up Fabrication and Manipulation of Lipid Nanotubes. International Journal of Nanoscience 1(5&6):465-469.

Sinha VR, Trehan A. 2003. Biodegradable microspheres for protein delivery. J Control Release 90(3):261-280.

Snow DM, Smith JD, Gurwell JA. 2002. Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. J Neurobiol 51(4):285-301.

Spencer T, Domeniconi M, Cao Z, Filbin MT. 2003. New roles for old proteins in adult CNS axonal regeneration. Curr Opin Neurobiol 13(1):133-139.

Steward O, Zheng B, Tessier-Lavigne M. 2003. False resurrections: distinguishing regenerated from spared axons in the injured central nervous system. J Comp Neurol 459(1):1-8.

Takami T, Oudega M, Bates ML, Wood PM, Kleitman N, Bunge MB. 2002. Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. J Neurosci 22(15):6670-6681.

Tang BL. 2003. Inhibitors of neuronal regeneration: mediators and signaling mechanisms. Neurochem Int 42(3):189-203.

Tarasenko YI, Gao J, Nie L, Johnson KM, Grady JJ, Hulsebosch CE, McAdoo DJ, Wu P. 2007. Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. J Neurosci Res 85(1):47-57.

Thomas CE, Ehrhardt A, Kay MA. 2003. Progress and problems with the use of viral vectors for gene therapy. Nature reviews 4(5):346-358.

Valentini RF, Aebischer P, Winn SR, Galletti PM. 1987. Collagen- and laminin-containing gels impede peripheral nerve regeneration through semipermeable nerve guidance channels. Exp Neurol 98(2):350-356.

Verdu E, Labrador RO, Rodriguez FJ, Ceballos D, Fores J, Navarro X. 2002. Alignment of collagen and laminin-containing gels improve nerve regeneration within silicone tubes. Restor Neurol Neurosci 20(5):169-179.

Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA. 1990. In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp Brain Res 81(2):283-287.

Vives E. 2003. Cellular uptake [correction of utake] of the Tat peptide: an endocytosis mechanism following ionic interactions. J Mol Recognit 16(5):265-271.

Vourc'h P, Moreau T, Arbion F, Marouillat-Vedrine S, Muh JP, Andres C. 2003. Oligodendrocyte myelin glycoprotein growth inhibition function requires its conserved leucine-rich repeat domain, not its glycosylphosphatidyl-inositol anchor. J Neurochem 85(4):889-897.

Walter J, Kern-Veits B, Huf J, Stolze B, Bonhoeffer F. 1987. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. Development 101(4):685-696.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. 2002a. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420(6911):74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z. 2002b. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417(6892):941-944.

Weidner N, Blesch A, Grill RJ, Tuszynski MH. 1999. Nerve growth factor-hypersecreting Schwann cell grafts augment and guide spinal cord axonal growth and remyelinate central nervous system axons in a phenotypically appropriate manner that correlates with expression of L1. J Comp Neurol 413(4):495-506.

Wells MR, Kraus K, Batter DK, Blunt DG, Weremowitz J, Lynch SE, Antoniades HN, Hansson HA. 1997. Gel matrix vehicles for growth factor application in nerve gap injuries repaired with tubes: a comparison of biomatrix, collagen, and methylcellulose. Exp Neurol 146(2):395-402.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L. 2002. Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277(36):32820-32829.

Woerly S, Doan VD, Evans-Martin F, Paramore CG, Peduzzi JD. 2001. Spinal cord reconstruction using NeuroGel implants and functional recovery after chronic injury. J Neurosci Res 66(6):1187-1197.

Woerly S, Doan VD, Sosa N, de Vellis J, Espinosa-Jeffrey A. 2004. Prevention of gliotic scar formation by NeuroGel allows partial endogenous repair of transected cat spinal cord. J Neurosci Res 75(2):262-272.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM. 2002. A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5(12):1302-1308.

Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. 1997. Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. J Neurocytol 26(1):1-16.

Xu XM, Zhang SX, Li H, Aebischer P, Bunge MB. 1999. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisected adult rat spinal cord. Eur J Neurosci 11(5):1723-1740.

Yick LW, Cheung PT, So KF, Wu W. 2003. Axonal regeneration of Clarke's neurons beyond the spinal cord injury scar after treatment with chondroitinase ABC. Exp Neurol 182(1):160-168.

Yoshii S, Oka M. 2001. Collagen filaments as a scaffold for nerve regeneration. J Biomed Mater Res 56(3):400-405.

Yoshii S, Oka M, Shima M, Taniguchi A, Akagi M. 2003. Bridging a 30-mm nerve defect using collagen filaments. J Biomed Mater Res 67A(2):467-474.

Yu X, Bellamkonda RV. 2003. Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Eng 9(3):421-430.

Yu X, Dillon GP, Bellamkonda RB. 1999. A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. Tissue Eng 5(4):291-304.

Yuan XB, Jin M, Xu X, Song YQ, Wu CP, Poo MM, Duan S. 2003. Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat Cell Biol 5(1):38-45.

Zelphati O, Szoka FC, Jr. 1996. Mechanism of oligonucleotide release from cationic liposomes. Proceedings of the National Academy of Sciences of the United States of America 93(21):11493-11498.

Zheng B, Atwal J, Ho C, Case L, He XL, Garcia KC, Steward O, Tessier-Lavigne M. 2005. Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proceedings of the National Academy of Sciences of the United States of America 102(4):1205-1210.

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M. 2003. Lack of enhanced spinal regeneration in Nogo-deficient mice. Neuron 38(2):213-224.

Zhou L, Shine HD. 2003. Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. J Neurosci Res 74(2):221-226.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D. 1998. Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Exp Neurol 154(2):654-662.

Zuo J, Neubauer D, Graham J, Krekoski CA, Ferguson TA, Muir D. 2002. Regeneration of axons after nerve transection repair is enhanced by degradation of chondroitin sulfate proteoglycan. Exp Neurol 176(1):221-228.