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Intrinsic and Extrinsic Factors for the Regeneration of Brainstem Axons after Spinal Cord Injury

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UNIVERSITY OF MIAMI

INTRINSIC AND EXTRINSIC FACTORS FOR THE REGENERATION OF BRAINSTEM AXONS AFTER SPINAL CORD INJURY

By

Ryan R. Williams

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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INTRINSIC AND EXTRINSIC FACTORS FOR THE REGENERATION OF
BRAINSTEM AXONS AFTER SPINAL CORD INJURY

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After traumatic injury, the successful regeneration of axons in the mammalian central nervous system (CNS) that leads to functional recovery will require a combination of therapeutic strategies. Following complete transection of the thoracic spinal cord, previous studies found the implantation of a bridge containing Schwann cells (SCs) and Matrigel promotes intraspinal but not supraspinal axon regeneration. Therefore, work in this dissertation sought to elucidate both intrinsic and extrinsic factors that enhance the regeneration of brainstem axons in a SC bridge. Adeno-associated viral (AAV) vector-mediated expression of green fluorescent protein (GFP) was utilized as a novel method for anterogradely tracing the regeneration of brainstem axons. AAV vectors also were used to determine if expression of a developmentally regulated transcription factor, MASH-1, allows mature brainstem neurons to regenerate axons more effectively. Compared to control animals, the expression of MASH-1 in brainstem neurons enhanced the regeneration of noradrenergic axons in a SC bridge and improved hindlimb joint movements. Surprisingly, in contrast to previous studies control animals also exhibited some brainstem axon regeneration. Analyses of the host spinal cord/SC bridge interfaces of control animals revealed variation from sharp to irregular boundaries. At the irregular
boundaries of the rostral interface in those animals, the regeneration of axons from noradrenergic neurons and other brainstem populations associated with the presence of long astrocyte processes entering the bridge. Furthermore, the total number of astrocyte processes that entered from the rostral and caudal interfaces associated with improvements in hindlimb joint movements. Previous studies of the SC bridge model implanted pre-gelled mixtures of SCs and Matrigel, in contrast to initially fluid mixtures implanted in this dissertation. Therefore, a direct comparison of these two bridge preparations was performed and demonstrated that initially fluid bridges exhibit increases in both brainstem axon regeneration and the number of astrocyte processes in the bridge. In summary, this work is the first to demonstrate that overexpression of MASH-1 can enhance CNS axon regeneration and that astrocyte processes are important for axon regeneration across a SC bridge. The combination of these intrinsic and extrinsic factors offers a new therapeutic strategy for promoting functional recovery after CNS injury.
Dedication

This dissertation is dedicated to my family, my friends, my mentors, The Regenerates, and my black Labrador Willy. Together, their patience, strength, guidance, and understanding afforded me the opportunity to complete this work.
Acknowledgments

This work was generously supported by the Miami Project to Cure Paralysis and Buoniconti Fund to Cure Paralysis. Dr. Damien Pearse and his team performed the complete transection and Schwann cell bridge implantation surgeries. Assistance with animal care was provided by the animal core facility at the Miami Project to Cure Paralysis and supervised by Drs. Alex Marcillo and Donna Avison. Generation of the adeno-associated viral vectors was in part carried out by the Viral Vector Core facility at the Miami Project to Cure Paralysis and the guidance of Dr. Bas Blits. Direction with microscopy was given by Dr. Beata Frydel in the Imaging Core at the Miami Project to Cure Paralysis. Assistance with immunohistochemistry and the generation of purified populations of Schwann cells was kindly provided by Raisa Puzis and Yelena Pressman, respectively. Drs. Caitlin Hill, Lawrence Moon, Martin Oudega, and Orion Furmanski provided invaluable scientific input, as well as Drs. Pat Wood and Jim Guest who also performed critical reading of chapters in this dissertation. Most importantly, Dr. Mary Bunge generously made the entire dissertation possible together with unsurpassed mentorship.
Preface

Due to my upbringing, I have always had a curiosity about the nature of consciousness and spirituality. Therefore, as an undergraduate at the University of California Santa Barbara in 1998, I majored in both Biopsychology and Zoology. While there, I was exposed first to neuroscience research by faculty who were members of the Center for Macular Degeneration, and I co-authored several publications concerning the development of the mammalian retina. As part of my studies, I took a course on oncogenesis where I read an article concerning the use of adenoviruses modified to deliver and induce the expression of transgenes in tumor cells. From that course I became enthralled with the newly emerging field of gene therapy and the potential for such technology to manipulate neuronal phenotypes.

After graduation, I spent several months volunteering around the globe with various international medicine groups, and while abroad I often spent free time reading articles concerning gene therapy. From that reading, I became increasingly wary of long-term consequences that may result from the use of gene therapy to produce inheritable genetic modifications. In that capacity, gene therapy has the potential to limit human diversity and variation in the genome, which are responsible for and facilitate the evolution of our species. Furthermore, unequal access to such technology may exponentially expand the current healthcare disparities between the world’s cultures and populations. Thus, upon returning to the U.S., I felt compelled to better understand and guide the use of gene therapy technologies with respect to neuroscience.
In seeking out a university in which I could study both gene therapy and neuroscience, as well as obtain further exposure to international medicine, I interviewed with faculty members at the University of Miami Miller School of Medicine. During the interviews I was introduced to my eventual dissertation mentor, Dr. Mary Bartlett Bunge at the Miami Project to Cure Paralysis as well as members of the newly organized group Project Medishare, who are responsible for numerous health clinics in Haiti. Based on those introductions I knew that together Dr. Bunge and Project Medishare would help fulfill my longer term career goals, so I enrolled in the M.D.-Ph.D. program at the University of Miami. Upon arriving at the University of Miami, I immediately began to study the use of gene therapy to promote axon regeneration in the spinal cord after injury, which evolved into my dissertation, “Intrinsic and extrinsic factors for the regeneration of brainstem axons after spinal cord injury”. The work toward this dissertation brought me great happiness as well as frustration, and ultimately became an obsession. Nevertheless, having been given such a great opportunity, I only hope that others will gain some insights, big or small, that will further treatment strategies for neural repair.
# Table of contents

List of figures and legends........................................................................................................... xi

List of abbreviations.................................................................................................................... xii

Publication notes ........................................................................................................................ xiv

## CHAPTER 1: INTRODUCTION ......................................................................................... 1

Overview .................................................................................................................................... 2

Spinal cord injury epidemiology ................................................................................................. 2

Spinal cord injury pathophysiology ........................................................................................... 3
  Immediate acute period ............................................................................................................. 3
  Intermediate sub-chronic period ............................................................................................... 5
    The inflammatory and immune response .............................................................................. 5
    Apoptosis, axonal sprouting, and dieback ........................................................................... 7
  Scar formation .......................................................................................................................... 10
    Extracellular matrix, cell-surface molecules, and soluble factors ..................................... 10
    Astrocytes ............................................................................................................................... 18
    Oligodendrocytes and Schwann cells .................................................................................... 24
    Neural progenitors ................................................................................................................ 25
    Meningeal fibroblasts ............................................................................................................. 28

Chronic period .............................................................................................................................. 31
  Wallerian degeneration ........................................................................................................... 31
  Cavity formation ...................................................................................................................... 33

Strategies to promote axon regeneration ..................................................................................... 34
  Extrinsic ..................................................................................................................................... 34
    Soluble growth factors .......................................................................................................... 34
    Overcoming inhibitory factors ............................................................................................ 35
  Transplantation ........................................................................................................................ 39
    Peripheral nerve .................................................................................................................... 40
    Extracellular matrix-like substrates ..................................................................................... 41
    Fibroblasts ............................................................................................................................... 42
    Schwann cells ......................................................................................................................... 43
    Embryonic and fetal tissue ...................................................................................................... 46
    Progenitor cells ...................................................................................................................... 47
    Olfactory ensheathing cells .................................................................................................... 49

C. Intrinsic .................................................................................................................................... 50
    Recreating the developmental phenotype .............................................................................. 51
    Conditioning lesions ............................................................................................................. 53
    Manipulations to neuronal somata ....................................................................................... 53
    Regeneration-associated genes ........................................................................................... 54
Second messengers ................................................................. 56
Rho/Rock ........................................................................ 57
Receptor tyrosine kinases .................................................... 60
Integrins and cell adhesion molecules .................................. 61
Axonal transport and translation .......................................... 62
Transcription factors .......................................................... 64
  Immediate early genes ...................................................... 64
  CREB ......................................................................... 65
  Krüppel-like factors ....................................................... 66
  basic helix-loop-helix .................................................... 67
  Retinoic acid receptors .................................................. 72
Epigenetic factors ................................................................ 73
Differentiation of noradrenergic brainstem neurons and their role in locomotion ......................................................... 74

CHAPTER 2: THE USE OF AN ADENO-ASSOCIATED VIRAL VECTOR TO ASSESS THE REGENERATION OF BRAINSTEM AXONS IN THE SPINAL CORD AFTER INJURY ................................................................. 78

Overview ............................................................................. 79

Background .......................................................................... 80

Methods ............................................................................... 82
  Experimental design ....................................................... 82
  Generation of adeno-associated viral (AAV) vectors ............ 83
  Stereotaxic injection of AAV ........................................... 83
  Generation of purified Schwann cells (SCs) ....................... 84
  Spinal cord transection and SC bridge implantation .......... 85
  Tissue processing and immunohistochemistry .................. 86
  Quantification of axon regeneration ................................. 87
  Statistics ......................................................................... 88

Results ................................................................................ 88
  AAV-GFP infects supraspinal brainstem neurons and labels their axons .......................................................... 88
  AAV-GFP anterogradely labels brainstem axons that regenerate into a SC bridge ............................................. 90
  AAV-GFP labeled axon-like structures are present in the caudal spinal cord distal to the SC bridge .................. 92

Discussion .......................................................................... 92
  The use of AAV-GFP as an anterograde tracer .................. 92
  The use of AAV-GFP to assess axon regeneration .......... 94
  The use of AAV as an intrinsic treatment strategy to express transgenes that regulate axon regeneration .... 97
CHAPTER 3: EXPRESSION OF THE DEVELOPMENTAL TRANSCRIPTION FACTOR, MASH-1, IN THE BRAINSTEM ENHANCES NORADRENERGIC AXON REGENERATION AND IMPROVES HINDLIMB JOINT MOVEMENTS AFTER COMPLETE SPINAL CORD TRANSECTION/IMPLANTATION OF A SCHWANN CELL BRIDGE

Overview, background, and results

Methods
- Experimental design and generation of AAV
- Stereotaxic injection of AAV, generation of purified SCs, and spinal cord transection and SC bridge implantation
- Tissue processing and immunohistochemistry
- Quantification of noradrenergic axon regeneration
- Assessment of hindlimb locomotor function
- Statistics

Discussion
- MASH-1 expression in noradrenergic brainstem neurons
- The expression of MASH-1 in the brainstem enhances the regeneration of noradrenergic axons into and across a SC bridge
- The expression of MASH-1 in the brainstem enhances hindlimb joint movements after complete transection of the thoracic spinal cord and implantation of a SC bridge

CHAPTER 4: ASSOCIATION OF GFAP POSITIVE PROCESSES ENTERING A SCHWANN CELL BRIDGE WITH THE REGENERATION OF BRAINSTEM AXONS AND IMPROVEMENTS IN HINDLIMB JOINT MOVEMENTS

Overview

Background

Methods
- Experimental design
- Generation of AAV vectors, stereotaxic injection of AAV, generation of purified SCs, and tissue processing and immunohistochemistry
- Spinal cord transection and initially fluid or pre-gelled SC bridge implantation
Quantification of axon regeneration and GFAP-positive processes .................................................................................... 148
Statistics.................................................................................................................. 149

Results ................................................................................................................................. 150
Implantation of a SC bridge alone is sufficient for brainstem axons to regenerate after complete transection of the thoracic spinal cord......................................................................................................................... 150
The presence of GFAP-positive processes is associated with increased regeneration of brainstem axons into a SC bridge ...... 151
Comparison of initially fluid with pre-gelled bridges of SCs and Matrigel ................................................................................................................................. 153
Association of GFAP-positive processes and hindlimb locomotion test scores ............................................................................... 154

Discussion ................................................................................................................................. 155
Brainstem axons regenerate into a SC bridge without an additional treatment strategy ........................................................................................................ 156
Brainstem axons regenerate in the peripheral and ventral regions of the polymer channel ...................................................... 157
Brainstem axons regenerate in areas of a SC bridge that contain macrophages ......................................................................................... 158
GFAP-positive processes entering the SC bridge define an interface that is permissive for axonal regeneration ......................... 161
The implantation of an initially fluid bridge of SCs and Matrigel enhances axon regeneration compared to a pre-gelled bridge ... 166
GFAP-positive processes define a SC bridge as permissive for improvements in hindlimb movements ...................................................... 169

Figures and legends .................................................................................................................. 171

CHAPTER 5: CURRENT AND FUTURE DIRECTIONS ....................................................................... 178
Overview ........................................................................................................................................ 179
Current treatment strategies ........................................................................................................ 179
Extrinsic treatment strategies ..................................................................................................... 184
Intrinsic treatment strategies ..................................................................................................... 186
REFERENCES .................................................................................................................................... 193
List of figures and legends

2.1: Experimental design ........................................................................................................... 99
2.2: AAV induces transgene expression in numerous brainstem neuron populations ......................................................................................................................... 101
2.3: In the brainstem, AAV-GFP only infects neurons ............................................................... 102
2.4: The stereotaxic injection of AAV-GFP into the brainstem functions to anterogradely trace brainstem axons in the thoracic spinal cord ................................... 104
2.5: The stereotaxic injection of AAV-GFP into the brainstem functions to anterogradely trace brainstem axons that regenerate into a SC bridge ........... 106
2.6: GFP may not be uniformly distributed along a regenerating axon ................................ 108
2.7: The stereotaxic injection of AAV-GFP into the brainstem followed by the complete transection of the thoracic spinal cord and implantation of a SC bridge labels structures in the caudal spinal cord ............................................ 110

3.1: Stereotaxic injection of AAV-GFP plus AAV-MASH-1 leads to expression of MASH-1 in adult noradrenergic brainstem neurons .............................. 134
3.2: Animals injected with AAV-MASH-1 compared to controls exhibit increased regeneration of noradrenergic axons in the SC bridge and improved scores in an open field locomotion test, the BBB ................................. 135
Supplementary 3.1: MASH-1 treated animals exhibit GFP expression in noradrenergic axons that regenerate into a SC bridge ......................................................... 137
Supplementary 3.2: Animals injected with AAV-MASH-1 compared to controls exhibit improved scores in an open field locomotion test, the BBB .. 139

4.1: The acute implantation of a polymer channel containing SCs and Matrigel is sufficient for brainstem axons to regenerate following complete transection of the thoracic spinal cord ........................................................ 171
4.2: Depending upon the interface, GFAP-positive processes cross the rostral host spinal cord/SC bridge interface together with brainstem axons .... 173
4.3: Increased brainstem axon regeneration is observed when more GFAP-positive processes enter a SC bridge .......................................................... 174
4.4: Acute implantation of an initially fluid bridge of SCs and Matrigel instead of a pre-gelled bridge improves the host spinal cord/SC bridge interface for growth of axons into the bridge ........................................ 175
4.5: Improvements in hindlimb joints movements are associated with increased numbers of GFAP-positive processes entering a SC bridge ........ 176
4.6: Acute implantation of fluid SC/Matrigel does not improve hindlimb locomotion compared to a pre-gelled SC/Matrigel bridge ............................... 177
List of abbreviations

AAV  Adeno-Associated Virus
AP-1  Activating Protein-1
ATF  Activating Transcription Factor
BDNF  Brain-Derived Growth Factor
bHLH  Basic Helix-Loop-Helix
CAM  Cell Adhesion Molecule
cAMP  Cyclic Adenosine Mono-Phosphate
CBP  CREB Binding Protein
cGMP  Cyclic Guanosine Mono-Phosphate
CNS  Central Nervous System
CNTF  Ciliary Neurotrophic Factor
CPG  Central Pattern Generator
CRE  cAMP Response Element
CREB  cAMP Response Element Binding Protein
CSPG  Chondroitin Sulphate Proteoglycan
CST  Cortical Spinal Tract
CTGF  Connective Tissue Growth Factor
DBH  Dopamine Beta Hydroxylase
db-cAMP  Dibutylryl cAMP
DNA  Deoxyribonucleic acid
DREZ  Dorsal Root Entry Zone
DRG  Dorsal Root Ganglion
ECM  Extracellular Matrix
EGF  Epidermal Growth Factor
FGF  Fibroblast Growth Factor
GAP-43  Growth-Associated Protein-43
GDNF  Glial Cell-Line Derived Neurotrophic Factor
GFAP  Glial Fibrillary Acidic Protein
GFP  Green Fluorescent Protein
GSK  Glycogen Synthase Kinase
HES  Hairy Enhancer of Split
Id  Inhibitor of Differentiation
IEG  Immediate Early Gene
KLF  Krüpple-Like Factor
JNK  c-Jun Terminal Kinase
MAG  Myelin-Associated Glycoprotein
MASH-1  Mammalian Achaete-Scute Homolog-1
MLC  Myosin Light Chain
MLR  Mesencephalic Locomotor Region
MMP  Matrix Metalloprotease
mRNA  Messenger Ribonucleic Acid
NCAM  Neural Cell Adhesion Molecule
NF  Neurofilament
NGF  Nerve Growth Factor
<table>
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<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>NT</td>
<td>Neurotrophin</td>
</tr>
<tr>
<td>OEC</td>
<td>Olfactory Ensheathing Cell</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte-Myelin Glycoprotein</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 Activated Kinase</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma-12</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>Phox</td>
<td>Paired Homeobox factor</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphoinositol-3-phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein Tyrosine Phosphatase</td>
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<tr>
<td>RA</td>
<td>Retinoic Acid</td>
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<td>RAR</td>
<td>Retinoic Acid Receptor</td>
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<tr>
<td>RAG</td>
<td>Regeneration Associated Gene</td>
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<td>RGC</td>
<td>Retinal Ganglion Cell</td>
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<td>ROCK</td>
<td>Rho-Associated Kinase</td>
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<tr>
<td>SC</td>
<td>Schwann cell</td>
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<tr>
<td>SCI</td>
<td>Spinal Cord Injury</td>
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<tr>
<td>SP-1</td>
<td>Serum Response Protein-1</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloprotease</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
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<tr>
<td>Trk</td>
<td>Tyrosine Kinase Receptor</td>
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Publication notes

Chapter 1 is being prepared for publication as two separate scientific journal reviews. The first will concern spinal cord injury pathophysiology and the second with concern extrinsic and intrinsic strategies for axon regeneration. The authors are Williams RR and Bunge MB.

Chapter 2 is being prepared for publication as a scientific journal article. The authors are Williams RR, Tresco P, Pearse DD, and Bunge MB.

Chapter 3 is being prepared for publication as a brief communication. Accordingly, due to the manuscript requirements, the overview, background, and results are presented as a single 1200 word section. However, additional methods and discussion sections are included in the chapter. The authors are Williams RR, Pearse DD, Bunge MB.

Chapter 4 is being prepared for publication as a scientific journal article. The authors are Williams RR, Pearse DD, and Bunge MB.
CHAPTER 1

INTRODUCTION
Overview

The evolving field of spinal cord injury (SCI) research is impressive, complex and multifaceted. Although the diversity of research in the field is ultimately inter-related, the scope of this introduction will be limited to a review of the known factors and mechanisms that regulate axon regeneration after SCI and when applicable to other central nervous system (CNS) injury models as well. This chapter begins by depicting numerous pathophysiological changes that may affect axon regeneration after SCI. These processes include extrinsic factors that are analogous to those present during development of the CNS, and some of which are deficient in the mature uninjured and injured CNS environment. With this background, the chapter then addresses therapeutic strategies that have been developed to surmount these extrinsic factors. Next the chapter provides an introduction to the mature phenotype of CNS neurons that is not capable of axon regeneration and addresses intrinsic treatment strategies that have been developed for the regeneration of axons after SCI. The chapter concludes with a focus on specific intrinsic factors that may regulate the regeneration of noradrenergic axons.

Spinal cord injury epidemiology

The inability of axons to regenerate robustly in the CNS has left millions of individuals who suffer from injury or neuronal disease without functional recovery. Of these, SCI alone afflicts approximately 2.5 million people world-wide and, according to the National Spinal Cord Injury Statistical Center, over 250,000 people in the United States, with about 12,000 new cases occurring each year.
SCIs are heterogeneous in cause and may lead to contusion, compression, penetration, shearing, laceration, or maceration of the parenchyma (Bunge et al., 1993). The socioeconomic impact of SCI is highly significant. In the United States these patients currently incur estimated lifetime health care costs of millions of dollars. These economic hardships are compounded further by the average age of injury occurring at 40 years, typically during the most productive period of a person’s life. In addition, ambulatory difficulties together with other disabilities place a strain on the individual, family, and friends. Such devastating effects of SCI were first described in Edwin Smith’s papyrus (2500 B.C.) which stated that SCI is “an ailment not to be treated” due to its severity and dismal recovery. Over the past 20-30 years, however, the current knowledge of SCI pathology has increased, and survival rate along with long-term management have greatly improved. Nevertheless, to date there are no fully restorative therapies for SCI, and this is, in part, due to the absence of robust elongation and guidance of injured axons. Thus, the mechanisms that regulate axon regeneration after SCI have been the foci of numerous studies and the treatment strategies that are being developed from this work will likely be translatable to other types of neuronal injury and disease as well.

**Spinal cord injury pathophysiology**

**Immediate acute period**

The pathology of SCI is subdivided chronologically into “immediate acute”, “intermediate sub-chronic”, and “chronic” periods. These distinct temporal periods have been extensively reviewed (Dumont et al., 2001; Norenberg et al.,
2004; Rowland et al., 2008) and will be used to outline the various pathophysiologies that effect axon regeneration in the CNS. The immediate acute phase begins with mechanical tissue destruction caused by the injury and the spinal cord is subject to vascular changes such as vasodilatation, vasogenic edema, disruption of the blood-brain barrier, and hemorrhage, as well as hypotension from the decreased sympathetic outflow of spinal shock (reviewed by Tator and Fehlings, 1991; Ditunno et al., 2004). These initial vascular changes not only produce ischemia, but also expose the “immune privileged” CNS to peripheral immune cells and, together with the resident immune cells, create an inflammatory response (Mautes et al., 2000). The inflammatory response starts with activation of the complement cascade (Anderson et al., 2004; Qiao et al., 2006) and an influx of neutrophils, which together with other dying cells release cytotoxic elements such as nitric oxide, free radicals, and proteases (Means and Anderson, 1983; Xu et al., 1990; Dusart and Schwab, 1994; Schnell et al., 1999; de Castro et al., 2004; Fleming et al., 2006). This early inflammatory response ensues together with hyperthermia (reviewed by Dietrich and Bramlett, 2007), progressive ischemia, nutrient deficiency, axonal swelling, disruption of myelin, and necrotic death. In addition, the vasogenic edema also gives rise to cytotoxic edema, which occurs mostly in astrocytes (Bullock et al., 1991); the various cation channels involved have been reviewed by Liang et al. (2007). Furthermore, the release of excitatory amino acids from dying neurons induces excitotoxic cascades affecting neighboring neurons and oligodendrocytes. These excitotoxic cascades produce increased levels of
intracellular calcium that results in lipid peroxidation/free radical injury as well as the activation of phospholipases, calpain proteases, and phosphatases, which together attack plasma membranes and other cellular components leading to further necrosis and apoptosis (reviewed by Park et al., 2004). Together, the immediate acute events initiate secondary tissue loss during the subsequent hours, days, and weeks, that results in a concentric expansion of the lesion penumbra and is often more devastating than the primary insult.

**Intermediate sub-chronic period**

*The inflammatory and immune response*

The intermediate sub-chronic phase begins a few days after injury and lasts for several weeks to a few months. During this time edema is resolved and the blood-brain barrier is slowly restored together with neovascularization (Casella et al., 2002; Loy et al., 2002). However, a cellular inflammatory response continues with an influx of lymphocytes and macrophages, as well as the activation of microglia (Popovich et al., 1997; Leskovar et al., 2000). The different activation states of these cells, and the environment in which they reside, lead to complex and multi-faceted inflammatory cascades that may be destructive and/or beneficial to axon regeneration and neural repair (reviewed by Bethea, 2000; Popovich and Longbrake, 2008). The adaptive immune cells, consisting of antigen presenting cells and T and B lymphocytes, serve both positive and negative functions for neural repair. These cells often persist at the lesion site indefinitely, where they increase circulating CNS-specific antibodies, mediate inflammatory signaling cascades, and induce cytotoxicity and/or promote
a “protective autoimmunity” (reviewed by Nguyen et al., 2002; Schwartz and Yoles, 2006; Ankeny and Popovich, 2009).

Controversy remains with respect to the role of innate immunity after SCI, given that CNS macrophages represent a heterogeneous population derived from monocyte precursors and resident microglia, and thus may have different “states” of activation depending upon their environment (reviewed by Donnelly and Popovich 2008; Laskin, 2009). Originally thought to function merely as scavengers of myelin and neuronal debris, microglia may be cytotoxic to neurons and glia due to the release of cytokines, nitric oxide, free radicals, glutamate, and matrix metalloproteases (MMPs; reviewed by Blight, 1992; Banati et al., 1993; Yong et al., 2001). Whereas MMP-2, -9, and -12 induce abnormal vascular permeability, which exacerbates the inflammatory and immune response resulting in apoptosis (Duchossoy et al., 2001a; Noble et al., 2002; Wells et al., 2003; Dang et al., 2008; Yu et al., 2008), they also mediate revascularization and wound healing of the lesion scar (Goussev et al., 2003; Hsu et al., 2006). MMPs are secreted as an inactive zymogen, subject to proteolytic activation by enzymes such as tissue plasminogen activator (tPA; reviewed by Romanic and Madri, 1994; Seeds et al., 1997). Although MMPs are counteracted by the expression of various tissue inhibitors of metalloproteases (TIMPs), only TIMP-1 is expressed transiently after SCI (Wells et al., 2003).

After SCI, the depletion of hematogenous macrophages promotes neuroanatomical repair (Popovich et al., 1999), and innate immunity activation by zymosan induces uncontrolled destruction of the spinal cord (Popovich et al.,
Furthermore, macrophages cause neurotoxicity damage to adjacent neuronal somata (Gensel et al., 2009), increase axonal die-back of dystrophic axons (McPhail et al., 2004; Horn et al., 2008), mediated by MMP-9 (Busch et al., 2009), and induce astrocytes to up-regulate putative inhibitory molecules (reviewed by Fitch and Silver, 1997b). In contrast, some of the beneficial characteristics of “activated macrophages” after SCI include being a source of growth factors, enzymes that degrade debris, and cytokines that promote the production of extracellular matrix necessary for axonal growth (reviewed by Lotan and Schwartz, 1994). In this capacity, non-CNS activated macrophages proximal (Lu and Richardson, 1991; Steinmetz et al., 2005) or distal (Gensel et al., 2009) to the somata of injured neurons may enhance the growth of axons in the spinal cord.

**Apoptosis, axonal sprouting, and dieback**

Given that most SCIs do not create a complete transection of the CNS parenchyma, bridges and rims of spared tissue remain at the lesion site following the primary insult. Due to the secondary injury events, this remnant tissue is subject to extensive apoptosis of neural cell types, including neurons (Feringa et al., 1985b; Liu et al., 1997; Springer et al., 1999; Lou et al., 1998; Yong et al., 1998; Grossman et al., 2001), microglia (Shuman et al., 1997) and oligodendrocytes around the site of injury as well as along the degenerating axonal pathways (Crowe et al., 1997; Yong et al., 1998; Abe et al., 1999; Li et al., 1999; Casha et al., 2001; Beattie et al., 2002). Furthermore, some atrophy and apoptosis of neuronal somata occur distal to the injury (Tetzlaff et al., 1991;
Kobyashi et al., 1997; Mori et al., 1997; Hains et al., 2003; Tang et al., 2004), and is dependent upon distance, in that fewer atrophied somata and apoptotic profiles are observed when the injury is more distal (Fernandes et al., 1999). Some corticospinal neurons may even present up to one year after injury (Feringa and Vahlsing, 1985; Nielson et al., 2010). Ultimately, the amount of spared tissue predicts the degree of functional recovery, and depending upon the axon population, as little as 5% of the remaining axons may be capable of producing significant behavioral effects (Blight et al., 1983; Noble and Wrathall, 1989; Basso et al., 1996).

Functional recovery and the formation of new synapses after SCI is, in part, due to local axonal sprouting from spared collaterals and an abortive axonal regeneration of severed axons (reviewed by Hagg and Oudega, 2006; Maier and Schwab, 2006). Axonal sprouting is a well established phenomenon, first described by Ramon y Cajal (1928) and detailed further by the pioneering studies of Windle and Chambers (1950), Windle et al. (1952), Clemente and Windle (1954), Liu and Chambers (1958), Matthews et al. (1979a, c), Guth et al. (1981, 1985), and Bunge et al. (1993, 1994). While some types of axons may sprout more readily than others, axonal sprouting has been observed from numerous populations including injured or uninjured intraspinal (Bareyre et al., 2004), corticospinal (Aoki et al., 1986; Li and Raisman, 1995; Hill et al., 2001; Bareyre et al., 2005; Hagg et al., 2005), serotonergic (Inman and Steward, 2003; Camand et al., 2004), catecholaminergic (Von Euler et al., 2002), and sensory neurons (McMahon et al., 1995). Although sometimes mistaken as axon regeneration,
collateral sprouting is distinct, and is often viewed as an extension of mechanisms that mediate synaptic plasticity (reviewed by Cafferty et al., 2008).

Axon regeneration, on the other hand, is typically long distance growth from injured axons, measured in millimeters to centimeters. In addition, to be considered regenerated, the axons must extend from the CNS to non CNS environment or take an unusual path through the tissue, as well as originate from the site of transection, and possess characteristic growth cone morphologies (Steward et al., 2003). During abortive axonal regeneration, the severed axon will typically grow only 10-50 micrometers before stopping, and this is likely due to transient changes in numerous intrinsic and extrinsic factors following injury.

Immediately after the axon is severed, calcium influx leads to resealing of the axonal tip through a vesicle-mediated process (Krause et al., 1994). From that point, significant alterations in the axonal morphology may occur (Bresnahan, 1978), such as formation of “dystrophic endings”, abnormally shaped ranging from small globular structures to huge multilobular sacs (Ramon y Cajal, 1928). Many dystrophic endings, dynamic in nature (Tom et al., 2004), contain organelles, such as mitochondria and smooth endoplasmic reticulum, and may be induced to regenerate again (Houlé, 1991; Li and Raisman, 1995). Distinct from the formation of dystrophic growth cones is growth cone collapse, where the ending appears more shrunken and often retracts, although these endings also can be induced to reform a growth cone during axon pathfinding (Fawcett et al., 1989; Burden-Gulley, 1995; Shibata et al., 1998). In addition, axonal dieback of hundreds of micrometers to millimeters is often observed after
Dieback may occur from axonal membrane damage induced by mechanical stretch (Shi and Pryor, 2002), excitotoxic- and inflammatory-mediated ionic imbalance/calcium influx and free radical-mediated lipid peroxidation (Shi et al., 2000), the progressive death of oligodendrocytes (Casha et al., 2001), or a process of Wallerian degeneration. Nevertheless, many dystrophic endings from supraspinal axons have been observed to remain close to the lesion site and do not significantly retract (Feringa and Vahlsing, 1985; Houlé and Jin, 2001).

**Scar formation: Extracellular matrix, cell-surface molecules, and soluble factors**

Together with the multifaceted inflammatory response and progressive apoptosis, there is formation of scar tissue composed of astrocytes, progenitor cells, inflammatory cells, meningeal fibroblasts (if the SCI penetrates the meninges), neuronal and myelin debris, and a complex extracellular matrix (ECM; reviewed by Reier and Houlé, 1988; Fitch and Sliver, 1997a; Fawcett and Asher, 1999; Silver and Miller, 2004). The scar is an evolving structure that is initially permissive for axonal growth as evident by local sprouting. However, the scar becomes progressively more inhibitory and functions to contain the injured CNS by forming a tight meshwork of astrocyte processes. Embedded within this meshwork are multiple layers of basal lamina or basement membrane that may be visualized by the electron microscope or the light/fluorescent microscope, respectively. These layers are similar to and become continuous with the glia.
limitans that surrounds the CNS (Kao et al., 1977a; Matthew et al., 1979a; Feringa et al., 1980, 1985a; Guth et al., 1981; Bernstein et al., 1985; reviewed by Brightman et al., 2002).

Sites of CNS injury have long been considered to form a physical barrier due to the presence of cellular debris, dense fibrous scar tissue, and cavities (Ramon y Cajal, 1928; Windle and Chambers, 1950; Windle et al., 1952; Clemente and Windle, 1954; Matthew et al., 1979b). However, careful analysis of SCIs, that contained relatively less physical impairments, suggested that chemical properties may have an equally important role in limiting axonal regeneration (Guth et al., 1981). This suggestion was elegantly confirmed through atraumatic microtransplantation experiments that created very small lesions and did not change in the physical architecture of the lesion site. In these experiments, adult neurons regenerated axons through the uninjured CNS only to halt in the ECM surrounding the lesion site (Davies et al., 1996; 1997; 1999).

The growth of axons in regions of uninjured CNS tissue contradicts the presence of numerous molecules both membrane-bound and in the ECM that are considered inhibitory to axonal growth; many of which are increased in expression after injury (reviewed by Grimpe and Silver, 2002; Liu et al., 2006; Busch and Silver, 2007). Thus, the white matter tracts may be permissive for some axonal growth when their architecture is intact and they are not expressing increased levels of inhibitory molecules. Noteworthy is that many of the inhibitory molecules, with increased expression after SCI, are also expressed during the development of the CNS, where they function to regulate axonal growth and
guidance (reviewed by Steindler, 1993; Clegg et al., 2003; Galtrey and Fawcett, 2007; Sherman and Back, 2008).

Early studies demonstrated that CNS myelin and white matter are inhibitory to neurite growth (Schwab and Thoenen, 1985; Schwab and Caroni, 1988; Caroni and Schwab 1988a, b; Crutcher, 1989; Fawcett et al., 1989; Savio and Schwab, 1989). Based on this work, three major substances were identified in CNS myelin that inhibit axonal growth: Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp; reviewed by Bandtlow, 2003; Filbin et al., 2003). Briefly, Nogo has three different isoforms, with NogoA being predominantly expressed in the CNS. MAG was the first axonal outgrowth inhibitor identified in both peripheral nervous system (PNS) and CNS myelin (McKerracher et al., 1994; Mukhopadhyay et al., 1994), and OMgp was the most recently identified (Kottis et al., 2002; Wang et al., 2002a). Interestingly, these three, structurally unique myelin-based inhibitors bind to the same receptor on growing axons, NgR (Josephson et al., 2002; Liu et al., 2002; Wang et al., 2002b), which lacks an intracellular domain and therefore requires the co-factor/receptor, p75 (Wang et al., 2002b; Wong et al., 2002; Yamashita et al., 2002), Lingo-1 (Mi et al., 2004), and TROY (Park et al., 2005; Shao et al., 2005). In addition, Nogo-A may inhibit axonal outgrowth through an integrin cell adhesion-dependent mechanism (Hu and Strittmatter, 2008). Another NgR-independent myelin-based inhibitor was recently discovered, termed repulsive guidance molecule (Schwab et al., 2005; Hata et al., 2006). Although the axonal growth inhibitors in myelin mediate contact-based growth cone collapse and
retraction (Fawcett et al., 1989; Bandtlow et al., 1993; Schnell and Schwab, 1993), they also have been considered as guidance cues that serve a “guard-rail” function. In this way, myelin may prevent growing axons from leaving undamaged and only partially denervated white matter tracts (reviewed by Raisman et al., 2004). This guard-rail function may explain, in part, the regeneration of axons in atraumatic white matter tracts (Davies et al., 1996; 1997; 1999).

There is an increase in expression of numerous cell-surface molecules that inhibit axonal growth in the scar after SCI (reviewed by Sandvig et al., 2004; Niclou et al., 2006; Bolsover et al., 2008). These include the ephrins and Eph receptors (Miranda et al., 1999; Bundesen et al., 2003; Willson et al., 2002, 2003; Irizarry-Ramirez et al., 2005) as well as the semaphorins (Pasterkamp et al., 1999, 2001; De Winter et al., 2002; Niclou et al., 2003; reviewed by Bareyre and Schwab, 2003). Ephrin ligands bind to Eph receptors, a large family of tyrosine kinase receptors divided into types A and B. The ephrin type A ligands are linked to the membrane through glycosylphosphatidylinositol, whereas the type B are transmembrane ligands. Semaphorins bind and signal through receptor complexes comprised of neuropilins and plexins expressed by neurons. After CNS injury, semaphorins may be secreted (Pasterkamp et al., 1999, 2001; De Winter et al., 2002) or membrane-bound (Moreau-Fauvarque et al., 2003). They also may be attractive or repulsive to axonal growth depending on the presence of other components in the ECM (Kantor et al., 2004).
There is an increase in expression of numerous ECM molecules in the scar after SCI that inhibit axonal growth, including tenascins (Zhang et al., 1995; Deckner et al., 2000; Tang et al., 2003; Apostolova et al., 2006), chondroitin sulfate proteoglycans (CSPGs; Pindzola et al., 1993; Lemons et al., 1999, 2001, 2003; Plant et al., 2001; Jones et al., 2002; 2003) and keratin sulfate proteoglycans (Jones and Tuszynski, 2002). The primary organizing polysaccharide of the ECM in the CNS, hyaluronic acid (reviewed by Frischknecht and Seidenbecher, 2008), forms a ternary complex with tenascin glycoproteins (reviewed by Faissner, 1997) and members of the lectican family of CSPGs. The four members of the lectican family, aggrecan, versican, neurocan, and brevican, that are associated with SCI, each contain a core protein with distinct chondroitin sulfate side chains (reviewed by Yamaguchi, 2000; Morgenstern et al., 2002). An additional CSPG found in the ECM of the scar is phosphacan, a messenger ribonucleic acid (mRNA) splice variant of the extracellular domain for receptor protein tyrosine phosphatase-β (PTP-β; Maurel et al., 1994). Finally, a structurally unique CSPG present within the scar is the integral membrane protein NG2 (reviewed by Levine and Nishiyama, 1996; Tan et al., 2005). NG2 has been considered by some groups as the major CSPG to inhibit axonal growth (Fidler et al., 1999; Jones et al., 2002), but this is debatable given its expression by cell types that appear to be permissive for axon regeneration.

The tenascins and the CSPGs normally function as perineuronal nets to stabilize synapses in the mature uninjured CNS (reviewed by Frischknecht and
Seidenbecher, 2008) and form boundaries for the developing nervous system; they are down-regulated in the mature CNS and re-expressed after injury by several cell types in the scar (reviewed by Steindler, 1993; Sherman and Black, 2008). They sequester soluble growth factors thereby limiting their diffusion and also serve as a reservoir (reviewed by Ruoslahti and Yamaguchi, 1991; Sugahara and Mikami, 2007). They bind to and inhibit axonal growth-promoting ECM and cell-surface molecules (Milev et al., 1994, 1996; Friedlander et al., 1994; Burg et al., 1996; Garwood et al., 2001), and induce intracellular signaling cascades similar to the inhibitory components in myelin (Yamashita et al., 1999, 2002; Wang et al., 2002b; Wong et al., 2002; Monnier et al., 2003; Schweigreiter et al., 2004). Fortunately, this complex inhibitory milieu of the scar is partially balanced by the presence of factors in the ECM that promote axonal growth.

One of the major neurite growth-promoting components of the ECM is the glycoprotein, laminin (Timpl et al., 1979; Manthorpe et al., 1983). Laminin self-assembles in a calcium-dependent manner to become a fundamental component of the basal lamina (reviewed by Timpl and Brown, 1996), is expressed abundantly after SCI, and is critical for axon regeneration (Liesi, 1985; Sosale et al., 1988; Risling et al., 1993; Frisén et al., 1995a; Grimpe et al., 2002). A second neurite growth component of the ECM, fibronectin, is also incorporated into the basal lamina (Carbonetto et al., 1982, 1983; Rogers et al., 1983; 1987). Furthermore, fibronectin is expressed after SCI (Farooque et al., 1992) and also is critical for axon regeneration (Tom et al., 2004). The last major axon growth promoting components of the ECM are the collagens. Types III and IV collagen
are critical components of the basal lamina involved in axonal regeneration in the PNS (Nathaniel and Pease, 1963; Ard et al., 1987). In the CNS, collagen is normally only found in the meninges, ependyma, and blood vessels (Shellswell et al., 1979; Azzi et al., 1989) but types I, III, and IV collagen become central components of the scar after SCI (Feringa et al., 1973, 1980, 1984; Mathews et al., 1979a; Tobin et al., 1979, 1980; Guth et al., 1981; Bernstein et al., 1985).

Protocollagen type IV self-assembles spontaneously into a dense non-fibrillar network that forms the backbone of the basal lamina (reviewed by Timpl and Brown, 1996). Collagen promotes neurite outgrowth from various types of neurons (Carbonetto et al., 1982; Kleitman et al., 1988). The early deposition of types I and III collagen as a more interstitial-like matrix correlates with early axonal sprouting after SCI (Mathews et al., 1979b; Risling et al., 1993; Zhang et al., 1997; Joosten et al., 2000; Iseda et al., 2003). However, continued expression of collagen over time, along with laminin and fibronectin, results in dense sheets and folds of basal lamina (Feringa et al., 1980, 1984; Berry et al., 1983; Bernstein et al., 1985; Loy et al., 2002). These basal lamina sheets may become surrounded by fibrillary bundles of collagen type I and III and/or CSPGs to create an ECM in the scar that is both a physical and molecular barrier to axonal growth (Guth et al., 1981; Liesi and Kauppila; 2002). Thus, the physical conformation of the ECM as well as the expression ratio of its numerous components determine the degree to which the scar impedes axonal growth (Meiners et al., 1995; Snow et al., 1996; Zhang et al., 1997; Liesi and Kauppila, 2002; Lemons et al., 2003). Noteworthy is that the structure of the ECM and
sheets of the basal lamina in the scar may be further modified by MMPs that degrade collagens, laminin, and fibronectin (de Castro et al., 2000; Duchossoy et al., 2001a; Bosman and Stamenkovic, 2003).

In addition to the ECM components, the scar is composed of numerous neurite growth-promoting factors that are either soluble or on the cell surface and thereby function through cell-cell and/or cell-ECM interactions. The soluble factors, together with their respective receptors, are transiently up-regulated during the intermediate sub-chronic period after SCI, and include nerve growth factors (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins-3 and -4 (NT-3, NT-4), glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF; Brunello et al., 1990; Reynolds et al., 1991; Frisén et al., 1992; Oyesiku et al., 1997; Zai et al., 2005). Some growth factor receptors have truncated intracellular domains, possibly functioning to diminish neurotrophin availability (King et al., 2000; Liebl et al., 2001). Cell-surface factors that mediate neurite growth include the integrins (Bixby et al., 1988; Drazba and Lemmon, 1990). Integrins function by forming various combinations of heterodimers that may also complex with adjacent cell surface proteins to regulate numerous cell to cell and ECM signaling pathways (reviewed by White et al., 2004; Denda and Reichardt, 2007). Integrins are suggested to be critical for axon regeneration (reviewed by Lemons and Condic, 2008), and serve important functions in determining cell-polarity (reviewed by Etienne-Manneville, 2008). Other molecules on the cell surface that promote axonal growth include the cadherins (Bixby et al., 1988; Bixby and Zhang, 1990; Drazba and Lemmon, 1990) and the
immunoglobulin superfamily of adhesion molecules (reviewed by Kamaguchi and Lemmon, 2000), such as neuronal cell adhesion molecule (NCAM; Bixby et al., 1987; 1988; Drazba and Lemmon, 1990) and L1 (Lagenaur and Lemmon, 1987; Drazba and Lemmon, 1990). The cell adhesion molecules (CAMs; reviewed by Zhang et al., 2008), soluble growth factors, as well as their respective receptors, are expressed by different cell types present in the scar after SCI, and together with the growth inhibitory components of the ECM and cell surface receptors, mediate, in part, the balance between axonal sprouting and abortive regeneration (McKeon et al., 1995; Lemons et al., 2003; Snow et al., 2003).

**Scar formation: Astrocytes**

Because the bulk of the CNS parenchyma as well as the scar tissue are composed of astrocytes, the majority of cell surfaces that are encountered by a regenerating axon are astrocytic. Astrocytes in and around the scar after SCI represent a diverse, heterogeneous, and highly plastic population that may promote and inhibit axon regeneration. The morphology of astrocytes is often indicative of their permissive or non-permissive nature for axonal growth. Permissive astrocytes are typically polarized, with linearly oriented processes and may resemble immature astrocytes or radial glia; these processes extend into the lesion site and are typically devoid of gap and tight junctions (Matthews et al., 1979b; Sims and Gilmore, 1983; Joosten and Gribnau, 1989; Kawaja and Gage, 1991; Suzuki and Raisman, 1992; Bunge et al., 1994; Davies, 1996; Krum and Rosenstein, 1999; Tom et al., 2004). Non-permissive astrocytes exhibit non-polarized morphologies similar to those of the glia limitans; they are arranged in a
tight meshwork of processes, running parallel to the boundary of the lesion, and are attached to one another by gap as well as tight junctions (Bernstein et al., 1985; Alonso and Private, 1993). Astrocytes may be classified as type-1 (A2B5 negative) astrocytes, that are mostly found within the CNS scar, or the type-2 astrocytes (A2B5 positive), which are not typically present near the lesion and are thought to die (Miller et al., 1986). Depending upon the distance from the lesion, many of the more proximate astrocytes become “reactive” in that they hypertrophy, increase arborization of their processes, and increase glial fibrillary acidic protein (GFAP) expression (Bignami et al., 1974; Bignami and Dahl, 1974; Barrett et al., 1981; reviewed by Eng et al., 2000); these may occur as early as 1 hour after injury (Hadley and Goshgarian, 1997). Noteworthy is that increased GFAP expression is also present in type-1 astrocytes adjacent to the glia limitans (Miller and Raff, 1984), suggesting a similar phenotype to those in the scar.

Although mature astrocytes are, in part, responsible for the formation of the scar after injury (Bernstein et al., 1985; reviewed by Reier et al., 1983), immature astrocytes do not produce a scar in the developing or mature CNS (Berry et al., 1983; Barrett et al., 1984; Smith et al., 1986; Smith and Silver, 1988; Smith et al., 1990; Smith and Miller, 1991). This is important given the significant cellular proliferation after SCI that may give rise to astrocytes with immature phenotypes. Thus, a given scar may be composed of astrocytes with a variety of phenotypes and functions, some of which are mature and may produce the ECM of the scar and others that do not.
Ablation of astrocytes (Bush et al., 1999) reveals numerous beneficial functions of these cells after injury. These functions include the restoration of the extracellular ionic environment, uptake of neurotransmitters, scavenging free-radicals, provision of nutrients, limiting the invasion of inflammatory cells, and secretion of growth factors and ECM components (reviewed by Muller et al., 1995; Renault–Mihara et al., 2008). Some of these beneficial functions may be mediated by reactive astrocytes whose molecular profile was reviewed by Eddleston and Mucke (1993) and Ridet et al. (1997). This profile includes increased expression of cell-adhesion molecules, components of the basal lamina, cytokines, growth factors, proteases, protease inhibitors, and inflammatory eicosanoids. Although these individual factors mediate numerous beneficial processes, some of them may ultimately limit axon regeneration.

In an attempt to elucidate the neuropathology of scar formation after SCI, numerous in vitro experiments have been developed to determine the role astrocytes play during neurite growth. However, it should be noted that the nature of primary tissue culture is somewhat analogous to injuring tissue and, therefore, makes it difficult to ascertain differences between uninjured and injured phenotypes. Nevertheless, cultures of immature astrocytes are a preferred substrate for neurite growth (Noble et al., 1984; Fallon et al., 1985a, b; Baehr and Bunge, 1990) as well as those prepared from the injured developing CNS (Rudge et al., 1989; Rudge and Silver, 1990; McKeon et al., 1991; Bahr et al., 1995). Astrocytes become less permissive after they have begun to mature and pass a “critical period” (Smith et al., 1986). Thus, cultures of mature CNS astrocytes
(Smith et al., 1990), derived from uninjured spinal cord (Zuo et al., 1998a), injured CNS scar tissue (Rudge et al., 1989; Rudge and Silver, 1990; McKeon et al., 1991; 1995; Bahr et al., 1995; Zuo et al., 1998a) or, more recently, following mechanical injury in vitro (Wanner et al., 2008), all exhibit limited neurite growth.

Astrocyte-mediated regulation of neurite growth is not primarily dependent upon a soluble factor, but rather the components they contribute to the ECM and molecules on their surface (Nobel et al., 1984; Geisert and Stewart, 1991; Smith-Thomas et al., 1994). Of these, neurites grow preferably on the surface of astrocytes rather than the ECM alone (Ard et al., 1991; 1993; Chalmers et al., 1996). Contact between neurites and astrocytes leads to a reduction in the amount of ECM (Ard et al., 1993), possibly mediated by astrocytic expression of MMPs that are increased after injury (Muir et al., 2002; Buss et al., 2007). This suggests that axonal die-back may have an indirect effect of increasing the ECM in the scar; reducing axonal dieback may allow more axons to be present at the lesion site which reduces scar formation and further enhances axon regeneration. The amount of ECM in the scar is important given that the primary difference between astrocyte cell lines that are permissive for neurite growth and those that are inhibitory is in the amount of ECM they generate, with more ECM being inhibitory (Smith-Thomas et al., 1994; Fok-Seang et al., 1995). This may be, in part, due to the ECM covering the growth permissive surface of astrocytes, because three-dimensional astrocyte cultures, which mimic the in vivo environment, exhibit ECM covering the astrocyte surfaces and are more inhibitory to neurite growth (Fawcett et al., 1989).
Astrocytes have the potential to express many of the known axonal growth inhibitors. Immature astrocytes express both tenascins and CSPGs in vitro (Johnson-Green, 1991; McKeon et al., 1991; 1995; Meiners et al., 1995) but not after injury in vivo (McKeon et al., 1991; Dow et al., 1994). Cultures of mature astrocytes, astrocyte cell lines, and reactive astrocytes make tenascins and CSPGs (McKeon et al., 1991; Fok-Seang et al., 1995; Canning et al., 1996; Zuo et al., 1998a; Fidler et al., 1999; Wanner et al., 2008). These molecules are also expressed by GFAP-positive astrocytes in the scar after injury of the mature spinal cord (Pindzola et al., 1993; Plant et al., 2001; Tang et al., 2003), especially NG2 (Fidler et al., 1999) and neurocan (Jones et al., 2003). After SCI, astrocytes also up-regulate receptors for axonal growth factors (Foschini et al., 1994) that may sequester soluble growth factors away from axons. Finally, reactive astrocytes also express EphB3 receptors (Miranda et al., 1999; Willson et al., 2003) as well as EphA3 and some ephrin A ligands (Willson et al., 2002; Irizarry-Ramirez et al., 2005).

Although astrocytes may express axonal growth inhibitors, they also have the potential to express factors that promote axonal growth (reviewed by Muller et al., 1995). In vitro, immature astrocytes may express cell surface molecules such as NCAM (Neugebauer et al., 1988; Chalmers, 1996), N-cadherin (Neugebauer et al., 1988), and β1 integrin (Neugebauer et al., 1988; Tomaselli et al., 1988). These factors are also expressed by astrocytes in the developing CNS (Smith et al., 1993). However, as astrocytes mature in vitro and in vivo, they down-regulate the expression of these cell surface molecules, with the
exception of β1 integrin (Smith et al., 1990; 1993). The maintenance of β1 integrin expression is relevant to findings in this dissertation, because β1 integrin is essential for establishing astrocyte polarity and process extension through the activation of the Rho GTPase, Cdc42 (Etienne-Manneville and Hall, 2001; Peng et al., 2008). Nevertheless, given the abundance of astrocytes, the majority of the cell surfaces in the mature uninjured CNS are not likely to express high levels of axonal growth promoting factors. In contrast to the cell surface molecules, mature uninjured astrocytes do make components of the ECM that promote axonal growth, such as laminin (Liesi et al., 1983; Ard and Bunge, 1988a, b; McKeon et al., 1991; 1995), fibronectin, and type IV collagen (McKeon et al., 1991; 1995). Furthermore, when mature astrocytes become reactive, they may increase the expression of ECM, cell surface, and soluble factors that promote axonal growth; these factors include laminin (Liesi et al., 1984; Frisén et al., 1995a), type IV collagen (Liesi and Kauppila, 2002; Iseda et al., 2003), NCAM (Chalmers et al., 1996), fibroblast growth factor (FGF; Liesi and Kauppila, 2002) and NGF (Schwartz and Nishiyama, 1994; Goss et al., 1998). Reactive astrocytes also make MMPs that break down inhibitory components of the scar such as the sheets of basal lamina and CSPGs, thus facilitating axonal growth (Duchossoy et al., 2001b; Muir et al., 2002; Larsen et al., 2003; Hsu et al., 2006) and revascularization (Goussev et al., 2003). In summary, a heterogeneous population of astrocytes exists around the lesion site following SCI; they mediate both permissive as well as non-permissive functions for axonal growth and these
functions are largely based on the nature of the ECM that they are, in part, responsible for creating.

**Scar formation: Oligodendrocytes and Schwann cells**

The extent of myelin debris has been considered as both a significant physical and chemical impediment to axonal growth, in part, because intact myelin tracts no longer remain to channel growing axons (Schwab and Schnell, 1991; Suzuki and Raisman, 1992; Davies et al., 1999; Pettigrew and Crutcher, 1999, 2001; Pettigrew et al., 2001). Although myelin is abundant in the CNS, few studies have conclusively demonstrated that oligodendrocytes increase the expression of the components in myelin that inhibit axonal growth after SCI (Guo et al., 2007; Dou et al., 2009). In contrast to the components in myelin, after CNS injury oligodendrocytes have been reported to increase expression of other axonal growth inhibitors, such as tenascin and CSPGs (reviewed by Morgenstern et al., 2002) as well as Sema4D (Moreau-Fauvarque et al., 2003). Despite these negative factors, oligodendrocytes also secrete neurotrophins (Condorelli et al., 1995) and have the potential to re-myelinate injured or regenerating axons (reviewed by Vicks et al. 1992). However, after SCI, Schwann cells (SCs) are probably the major cell type responsible for myelination (Blakemore, 1975; Mathews et al., 1979a, b; Bunge et al., 1994, Beattie et al., 1997). SCs may infiltrate from the PNS along damaged roots (sensory axons) or blood vessels, in part, due to chemoattractants released by macrophages (Jasmin et al., 2000), and they were recently reported to arise from endogenous CNS progenitors (Zawadzka et al., 2010). In addition to remyelination, SCs may serve numerous
other benefits, such as the secretion of neurotrophins and components of the basal lamina. These benefits are discussed in the section concerning SC transplantation as an extrinsic treatment strategy (Chapter 1, pages 43-46).

**Scar formation: Neural progenitors**

The identification of endogenous multi-potent, self-renewing, neural progenitors in the mature uninjured and injured mammalian CNS was a major advancement for neural regeneration and repair (Altman and Das, 1965; Reynolds and Weis, 1992). Although these progenitor cells maintain the capacity to generate both neurons and glia in specific locations such as the hippocampus and sub-ventricular zone (Levison and Goldman, 1993; Lois and Alvarez-Buylla, 1993; Gage et al., 1995a; Palmer et al., 1997; Eriksson et al., 1998; Shihabuddin et al., 2000), they only give rise to glia in both the uninjured and injured spinal cord (Frisén et al., 1995b; Johansson et al., 1999; Cao et al., 2001, 2002; Vroemen et al., 2003; Attar et al., 2005; Azari et al., 2005; Mothe and Tator, 2005; Zai and Wrathall, 2005; Horky et al., 2006). Analogous to neural progenitors during development, these cells are typically identified by the expression of the intermediate filament protein, nestin (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990). A very small and quiescent population of these cells is present in the uninjured spinal cord associated with vascular endothelial cells, white matter, and the ependyma (Frisén et al., 1995b; Weiss et al, 1996; Horner et al., 2000). A significant cellular proliferation is well known to occur in the spinal cord after injury (Adrian and Walker, 1962; Mathews et al., 1979a; Vaquero et al., 1981; Wallace et al., 1987).
that may, in part, be mediated by growth factors such as NGF, CNTF, TGF-β, and platelet-derived growth factor (PDGF; Cattaneo and McKay, 1990; Lindholm et al., 1992; Ip et al., 1993; Johe et al., 1996); bFGF and epidermal growth factor (EGF) remain the most potent for proliferation and maintenance of nestin expression as well as the multipotent state (Cattaneo and McKay, 1990; Lewis et al., 1992; Reynolds and Weiss, 1992; Wolswijk and Noble, 1992; Shihabuddin et al., 1997; Weiss et al., 1996; Kojima and Tator, 2000, 2002).

Nestin expression is often co-localized with GFAP-positive cells and their long processes derived from the ependyma or dedifferentiating/pre-existing GFAP-positive astrocytes (Johansson et al., 1999; Namiki and Tator, 1999; Kojima and Tator, 2000, 2002; Yamamoto et al., 2001; Shibuya et al., 2002; Walder et al., 2003; Attar et al., 2005; Mothe and Tator, 2005; Horky et al., 2006). Low-density purified cultures of astrocytes increase nestin expression when they are mechanically injured and these nestin-positive astrocytes are permissive for neurite growth (Frisén et al., 1995b). Thus, it appears that after SCI some astrocytes de-differentiate to an earlier developmental state that may facilitate axonal growth.

One of the first markers identified for immature glia was the intermediate filament protein, vimentin (Dahl et al., 1981; Schnitzer et al., 1981). During gliogenesis, vimentin is first expressed together with nestin on radial glial precursors that are capable of generating both neurons and glia (reviewed by Morest and Silver, 2003; Malatesta et al., 2008). Vimentin expression by immature glia of the developing spinal cord also correlates with the establishment
of axonal tracts and remains in a few longitudinally oriented astrocytes of the white matter in the adult (Hutchins and Casagrande, 1989; Brusco et al., 1995). Vimentin expression is increased after SCI (Mikucki and Oblinger, 1991; Pindzola et al., 1993; Farooque et al., 1995; Baldwin et al., 1998), and co-localizes with GFAP- or nestin-positive cells (Shibuya et al., 2003). A specific type of vimentin-positive progenitor cell, the tanycyte, is present along the central canal, throughout the length of the cord (Bruni and Reddy, 1987), and gives rise to both ependymal cells and astrocytes (reviewed by Wittkowski, 1998). Tanycytes have been shown to support axonal growth from numerous neuronal cell types, including those of the catecholaminergic and serotonergic lineages (Chauvet et al., 1995, 1996, 1998; Ridet et al., 1996) as well as sprouting and/or regeneration of spinal cord axons (Prieto et al., 2000; West et al., 2001; Dervan and Roberts, 2003).

Lastly, the CNS contains a bi-potent progenitor cell first described by Raff et al. in 1983. Distinct from the progenitor cells described above, the O-2A progenitor cell only differentiates into both astrocytes and oligodendrocytes after isolation from the adult spinal cord (Engel and Wolswijk, 1996). These cells may be identified by the expression of the type-2 astrocyte marker, A2B5, as well as by expression of the CSPG, NG2, and are therefore often referred to as NG2 cells (Gallo et al., 1987; Levine and Stallcup, 1987; Wolswijk and Noble, 1989). NG2 cells have two distinct morphologies, the first resembling immature astrocytes with a bipolar morphology, but no expression of astrocytic markers. The second, is a stellate, multipolar morphology, resembling immature
oligodendrocytes, but lacks the expression of mature oligodendrocyte markers (reviewed by Levine et al., 2001; Horner at al., 2002; Wang and He, 2009). NG2-positive cells are well known to contribute to scar formation after SCI (Keirstead et al., 1998; McTigue et al., 2001; Jones et al., 2002; Rosenberg et al., 2005). They secrete CSPGs (Asher et al., 2000, 2002) and they give rise to a portion of the nestin-positive population after various CNS injuries (Johansson et al., 1999; Palmer et al., 1999; Horner et al., 2000; Zai and Wrathall, 2005; Horky et al., 2006). In addition to generating immature astrocytes, NG2-positive cells also promote axon growth in the injured spinal cord that may be, in part, due to the reduction of macrophage-induced axonal dieback (Busch et al., 2010). One final note is that as NG2 has been reported to be expressed by immature SCs (Schneider et al., 2001). Therefore, co-localization with other progenitor cell markers, such as the transcription factor Olig2, may be necessary for proper identification of the NG2-positive progenitors that populate the lesion site. In contrast to Schneider et al. (2001), several other groups suggest that NG2 is only expressed by fibroblasts and satellite cells, but not s100-positive cells or SCs in the developing, adult, and injured PNS (Morgenstern et al., 2003; Rezajooi et al., 2004). Therefore, NG2-positive cells in sites of SCI are probably CNS derived O-2A-like progenitors and/or infiltrating fibroblasts.

**The Scar: Meningeal fibroblasts**

A specialized class of fibroblast, the meningeal fibroblast, surrounds the brain, spinal cord, and large blood vessels and often invades the lesion site after SCI. Meningeal fibroblasts secrete high levels of laminin, fibronectin, and types I,
III, and IV collagen, and thus contribute to the formation of dense basal lamina sheets in the scar (Sievers et al., 1994; Hirsch and Bahr, 1999). Furthermore, they express inhibitory components that are incorporated into the scar, such as tenascins, CSPGs (Ajemian et al., 1994; Ness and David, 1997; Hirsch and Bahr, 1999; Jones et al., 2002; Shearer et al., 2003) and semaphorins (Pasterkamp et al., 1999; De Winter et al., 2002a; Niclou et al., 2003). The expression of these ECM components and their proliferation are mediated, in part, by integrin signaling (reviewed by Chiquet et al., 2003). Reductions in meningeal cells (Li and David, 1996), their TGF-α- and β-dependent proliferation (Logan et al., 1994, 1999a, b; Moon and Fawcett, 2001; Lagord et al., 2002), and/or production of connective tissue growth factor (CTGF; Grotendorst, 1996; Duncan et al., 1999; Schwab et al., 2001) decrease basal lamina formation. Lastly, fibroblasts also express ephrins that function to inhibit neurite growth (Bundesen et al., 2003). Together these cell-surface and ECM molecules make meningeal fibroblasts less permissive for neurite growth than astrocytes and, when neurites do grow, the neurites are often found in fascicles limiting their exposure to the environment (Nobel et al., 1984; Ness and David; 1997; Hirsch and Bahr, 1999; Shearer et al., 2003; Wanner et al., 2008).

Perhaps the most prominent cell-cell interaction that limits axon regeneration after SCI occurs between meningeal fibroblasts and astrocytes. The interaction of these cells largely contributes to structures within the scar that resemble the glia limitans (reviewed by Hermanns et al., 2001; Shearer and Fawcett, 2001) and, not surprisingly, this interaction also regulates the formation
of the glia limitans during CNS development (Lyser, 1972; Kusaka et al., 1984; 1985; Pehlemann et al., 1985; Sievers et al., 1985). Contact between astrocytes and meningeal fibroblasts results in their segregation from each other (Abnet et al., 1991; Franklin et al., 1992; Struckhoff, 1995; Hirsch and Bahr, 1999; Shear et al., 2003; Wanner et al., 2008). Furthermore, their interactions promote the expression of tenascins and CSPGs (Ajemian et al., 1994; Ness and David, 1997; Shearer et al., 2003).

Interestingly, when in contact with meningeal cells, astrocytes are found in both permissive and non-permissive morphologies. In this way, some astrocytes form a border with a thin meshwork of their processes adjacent to the meningeal cells, and some astrocytes extend processes underneath the meningeal cells (Struckhoff, 1995; Ness and David; 1997; Hirsch and Bahr, 1999; Shearer et al., 2003). Despite the presence of astrocyte processes, neurite outgrowth is only observed from meningeal cells onto astrocytes, and not from astrocytes onto meningeal cells (Ness and David, 1997; Hirsch and Bahr, 1999). However, neurites may be enticed to leave the growth-permissive astrocyte surfaces. For example, neurite outgrowth may be induced across co-cultures by the application of antibody-mediated blockade of NG2 or modifications to intrinsic states such as Rho or cyclic adenosine monophosphate (cAMP) signaling (Shearer et al., 2003). In vivo, meningeal fibroblasts in scars segregate from astrocytes, with the exception of immature astrocytes that express vimentin (Abnet et al., 1991). This segregation is also associated with increased astrocytic expression of CSPGs (Tang et al., 2003) and may be mediated by
astrocyte expression of EphB2 and meningeal fibroblast expression of Ephrin-B2 (Bundesen et al., 2003). Noteworthy is that an extensive scar does not form when the spinal cord is injured during development (Barrett et al., 1984), and this may be due to a lack of Eph2B expression by immature astrocytes. Thus, the interaction between immature astrocytes and meningeal cells appears to be distinct from their interactions in the adult.

**Chronic period**

**Wallerian degeneration**

After SCI, there is a slow anterograde and retrograde degeneration of axons in the long tracts, termed Wallerian degeneration. Although this process begins immediately after SCI, the degeneration is observed for months to years (reviewed by Vargas and Barres, 2007) and can be assessed in patients using magnetic resonance (Becerra et al., 1995). First described in the PNS by Waller (1850) and in CNS axonal tracts by Ramon y Cajal (1928), electron microscope studies later characterized this degenerative process in the spinal cord as distorted and fragmented myelin sheaths, beading and swelling of axons, leading to granular disintegration of the axonal cytoskeleton, a reactive astrocyte response, and a microglial-mediated phagocytosis of myelin and axonal debris (Bignami and Ralston, 1969; Bignami et al., 1981). Longer and thicker axons tend to degenerate more slowly than smaller, shorter axons (Lubinska, 1977). Although calcium-activated caspases are present in axons after injury (Buki et al., 2000), the beading/swelling of the axon appears to be a calcium/apoptotic-independent process, whereas the granular disintegration of the axonal
cytoskeleton is calcium-dependent (Schlaepfer and Bunge, 1973; Finn et al., 2000; Zhai et al., 2003). For a period of time after axotomy, these axon-like structures remain functional, in that they have intact plasma membranes, contain neurofilaments, and have an active metabolism that preserves physical integrity (Bignami et al., 1981; Deckwerth and Johnson, 1994).

Given the temporary viability of the remnant axon structures, the axonal degeneration is thought to be, in part, an active process mediated by intrinsic mechanisms (reviewed by Raff et al., 2002). This notion is supported by the identification of the WldS mutant mouse that exhibits delayed Wallerian degeneration in the CNS (Lunn et al., 1989; Perry et al., 1991). The autosomal dominant WldS gene encodes for a chimeric ubiquitin assembly protein linked to nicotinamide adenine dinucleotide transferase (Coleman et al., 1998; Conforti et al., 2000; Araki et al., 2004). This protein localizes to the nucleus and is not detectable in the axon, which indicates an indirect mechanism of action (Mack et al., 2001; Fang et al., 2005) that delays anterograde but not retrograde degeneration (Cheng and Burke, 2010). Nevertheless, Wallerian degeneration may not be entirely dependent upon intrinsic mechanisms and other cellular or extrinsic factors, such as activated macrophages, are likely required. The absence of such extrinsic factors may account for the slow nature of this process in regions of the CNS distal to the site of injury.

The slow degeneration of axons perpetuates together with progressive apoptosis of oligodendrocytes (Crowe et al., 1997; Dong et al., 2003), although which one comes first remains unclear. Myelin sheaths are thought to protect
degenerating axons from degradation by resident microglia (Perry et al., 1987; George and Griffin, 1994). However, the long-term presence of myelin debris suggests that the resident microglial response is relatively slow and few hematogenous macrophages are involved (Bignami and Ralston, 1969; Perry et al., 1987; George and Griffin, 1994; Koshinaga and Whittemore, 1995; Wang et al., 2009). As the degeneration progresses, the tissue is replaced by a glial scar (Puckett et al., 1997; Wang et al., 2009) that lags behind the microglial response (Basiri and Doucette, 2010). Sites of Wallerian degeneration also contain nestin- (et al., 1995b) and vimentin- (Dahl et al., 1981; 1982) positive cells, suggesting the presence of glial progenitors that may offer some axonal growth promoting components.

Cavity formation

In many species, cavities may form in the weeks to months following SCI and are visible by magnetic resonance. Occasionally, cavities predispose a progressive syringomyelia, thereby compressing adjacent parenchyma, but more importantly cavities create physical barriers to axon regeneration. The walls of cavities may be composed of astrocytes, ependymal cells, fibroblasts, dense collagen, and the interiors may contain trabeculae, fluid, and macrophages (Kao et al., 1977b; Guth et al., 1981; Wozniewicz et al., 1983; Wallace et al., 1987; Hill et al., 2001). Their formation is due to macrophage activity (Bush et al., 1999; Fitch et al., 1999), disruption of the vasculature (Rooney et al., 2009), and/or Wallerian degeneration in white matter (Zhang et al., 1996; Zhang and Guth,
1997). Many of the cellular and substrate transplantation strategies discussed below are designed to eliminate or minimize the formation of cavities.

**Strategies to promote axon regeneration**

**Extrinsic**

**Soluble growth factors**

The “lack of trophic factor hypothesis” for the poor axonal regeneration observed from CNS neurons was initially proposed by Ramon y Cajal (1928). The mature spinal cord does not express soluble trophic molecules associated with axonal growth to the same extent as it does during development. Although a transient increase in many of these factors does occur after SCI, it is mostly short-lived. Therefore, numerous investigators, using a variety of different experimental models of SCI, have either exogenously applied soluble trophic factors (Fernandez et al., 1990; Schnell et al., 1994; Xu et al., 1995b; Ye and Houlé, 1997; Bradbury et al., 1999; Oudega and Hagg, 1996, 1999; King et al., 2000; Namiki et al., 2000; Bregman et al., 2002; Ramer et al., 2002) or transplanted various cell types that were genetically modified to secrete them (Nakahara et al., 1996; Tuszynski et al., 1996, 1998; Romero et al., 2001; Menei et al., 1998; Weidner et al., 1999a; Hurtado et al., 2006; Golden et al., 2007). In each of these studies, the authors reported the soluble trophic factors to enhance axonal growth. Furthermore, the factors appeared to be most beneficial when applied during the intermediate sub-chronic period (Bregman et al., 2002). Thus, the application of soluble trophic factors or cells that express them will likely be a
necessary component for combination therapeutic strategies designed to promote axon regeneration.

**Overcoming inhibitory factors**

Numerous extrinsic strategies may overcome the various axonal growth-inhibitory molecules. These include strategies that neutralize the inhibitory components in myelin with function-blocking antibodies (Caroni and Schwab, 1988a, Savio and Schwabb, 1989). The application of these antibodies in partial and complete transection models of SCI enhances growth of corticospinal and brainstem axons and modestly improves recovery of function (Schnell and Schwab, 1990, 1993; Bregman et al., 1995; Guest et al., 1997b; Brosamle et al., 2000; Merkler et al., 2001). In addition to function-blocking antibodies, the use of an antagonistic peptide to Nogo-66 receptor improves corticospinal axon growth and functional recovery (GrandPre et al., 2002; Li and Strittmatter, 2003). Another strategy utilizing Sialidase, an enzyme that cleaves the extracellular portion of the receptor, NgR, increases axon regeneration into peripheral nerve grafts (Yang et al., 2006). Noteworthy is that Nogo, NgR, and MAG knock-out mice, as well as mutant p75 mice exhibit conflicting results after SCI (Bartsch et al., 1995; Kim et al., 2003, 2004; Simonen et al., 2003; Zheng et al., 2003, 2005; Song et al., 2004; Dimou et al., 2006), suggesting that there may be differences in the genetic background of the mice or that the factors in myelin that inhibit axonal growth are only partly responsible for the lack of regeneration after SCI.

Another promising strategy to overcome inhibitory factors for axonal growth is the use of enzymes to degrade specific components of the ECM.
Enzymes with wide specificity, such as trypsin, result in extensive hemorrhaging due to disruption of the basement membrane around the vasculature (Feringa et al., 1979; Guth et al., 1980). However, specific enzymes such as chondroitinase, which degrade CSPGs, enhance axonal growth in the CNS (Moon et al., 2001). The direct application of chondroitinase to sites of SCI, increases sprouting and/or regeneration of sensory, corticospinal, and brainstem axons, as well as improves post-synaptic activity and functional recovery (Bradbury et al., 2002; Steinmetz et al., 2005; Barritt et al., 2006; Tester and Howland, 2006). The use of chondroitinase also enhances the regeneration of axons across a SC graft in a lateral cord hemisection (Chau et al., 2003) and into peripheral nerve grafts implanted in the spinal cord (Yick et al., 2003; Houlé et al., 2006; Tom and Houlé, 2008). The benefits of chondroitinase appear to be greatest when applied acutely after injury (Garcia-Alias et al., 2008) or when hyperemia is reduced (Tester et al., 2007). Because regions of digested CSPGs may still be inhibitory (Lemons et al., 2003; Tom et al., 2004; Ughrin et al., 2003), other strategies to remove CSPGs may be more effective (Grimpe and Silver, 2004). For example, the down-regulation of mRNA for a CSPG synthetic enzyme increases the regeneration of axons into a peripheral nerve graft (Hurtado et al., 2008). Thus, the application of enzymes that degrade specific components of the ECM, and/or the manipulation of synthetic enzymes for those components, will likely be useful extrinsic treatments to induce the regeneration of axons after SCI.

In addition to chondroitinase, other types of enzymes, such as MMPs and tPA, have very specific and localized effects on the ECM; they appear to clear a
passage for axonal growth in CNS development as well as after injury (reviewed by Seeds et al., 1997; Pizzi and Crowe, 2007; Yong et al., 2007; Agrawal et al., 2008). In vitro, MMP-2 is transported to the growth cone of dorsal root ganglion (DRG) neurons where it is secreted and degrades CSPGs (Zuo et al., 1998b). MMP-2 and -9 are expressed in regenerating PNS axons and increase the neurite-promoting potential of SC basal lamina (Ferguson and Muir, 2000; Shubayev and Myers, 2002). MMPs cleave and inactivate NgR (Walmsley et al., 2004) as well as p75 (Weskamp et al., 2004). MMPs also activate and release cytokines as well as growth factors (reviewed by Fowlkes and Winkler, 2002). MMP-9 improves NGF-induced neurite elongation of pheochromocytoma-12 (PC12) cells (Shubayev and Myers, 2004) and MMP-3 is required for PC12 neurites to penetrate solidified Matrigel (Nordstrom et al., 1995). Although MMP-9 is expressed at low levels in uninjured anterior horn neurons (Nobel et al., 2002), after SCI, the few axons that do sprout into the scar express MMP-2 and -9 (Duchossoy et al., 2001b). Following the implantation of peripheral nerve into the vitreous, MMP-2 and -9 are expressed in the growth cones of regenerating retinal ganglion cell (RGC) axons as well as adjacent glia (Ahmed et al., 2005). The transplantation of fibroblasts, induced to express MMP-3 after spinal cord contusion, enhances the growth of supraspinal axons as well as improves recovery of locomotion (Pizzi and Crowe, 2006). Progenitor cells, transplanted after SCI, reduce scar formation in an MMP-2-dependent manner (Veeravalli et al., 2009). Finally, tPA is required for DRG neurons to grow into the CNS after conditioning lesions (Minor et al., 2009), suggesting an important role for this
serine protease in activating MMP zymogens. Together, these studies further emphasize the importance of enzymes for modifying the extracellular environment and thereby promoting axonal growth.

Given that the dense basal lamina sheets in the scar serve as a backbone upon which additional inhibitory factors are incorporated, other extrinsic strategies are designed to limit the formation of these collagenous basement membranes (reviewed by Klapka and Muller, 2006; Brazda and Muller, 2009). This may be accomplished indirectly through the use of duraplasty to prevent the invasion of meningeal fibroblasts after SCI (Iannotti et al., 2006). In addition, manipulations to the inflammatory and cytokine signaling cascades may be useful. For example, application of the anti-inflammatory agents cyclophosphamide and suramin reduce the amount of basal lamina after SCI (Feringa et al., 1985a) and abrogate the glial response (Di Prospero et al., 1998), respectively. Expression of the proteoglycan, decorin, also limits the production of basement membrane and increases axon growth, presumably through binding and sequestering the cytokine, TGF-β1 (Logan et al., 1999a; Davies et al., 2004). However, decorin may induce axon growth by acting on the neurons themselves (Minor et al., 2008). Nevertheless, direct reductions in TGF-β1 decrease basement membrane formation and increase axonal growth as well as functional recovery (Logan et al., 1994; 1999b; Moon and Fawcett, 2001; King et al., 2004). While manipulations to inflammatory and cytokine signaling cascades are promising, a more direct strategy to reduce the formation of basal lamina is to inhibit the synthesis of the primary component, collagen type IV. This may be
accomplished by local application of an iron chelator to inhibit prolyl 4-hydroxylase, the key enzyme in collagen biosynthesis. Although the immediate injection of the iron chelator alone does not diminish the amount of basement membrane (Hermanns et al., 2001) or enhance corticospinal axon growth (Weidner et al., 1999b), the amount of basement membrane in the scar is dramatically reduced when the iron chelator is combined with the inhibition of fibroblast proliferation (Grotendorst, 1996; Duncan et al., 1999; Hermanns et al., 2001; 2006). This combination results in long-distance axonal growth from corticospinal (Klapka et al., 2005) and brainstem (Schiwy et al., 2009) neurons after dorsal hemisection of the cord. Therefore, in order to limit scar formation after SCI, multiple therapies should be combined, such that the synthesis of basal lamina is reduced at both the cellular and molecular level.

**Transplantation**

Guth et al. (1985) emphasized that injured CNS axons require a substrate or cellular terrain for their regeneration. In addition, the extracellular environment of the lesion site consists of numerous impediments to axonal growth. Therefore, the implantation of permissive bridges or substrates to circumvent and/or modify the injury site may promote axon regeneration. With respect to SCI, numerous transplantation strategies have been studied (reviewed by Bunge and Pearse, 2003; Reier, 2004; Tetzlaff et al., 2010). Few of these studies, however, have observed significant regeneration of axons beyond the bridge or transplant, and the success of these transplantation experiments appears to be dependent upon
combination strategies or the nature of the host spinal cord/implant interface that will be discussed in a subsequent section.

**Transplantation: Peripheral nerve**

The raison d’ etre for transplantation strategies after SCI was provided by Kao et al. (1977a) and Richardson et al. (1980; 1982), following upon early work by Ramon y Cajal’s student Tello (1928). In this more recent seminal work to determine if CNS neurons maintained at least some intrinsic capacity to regenerate axons, Richardson et al. (1980) demonstrated that sciatic nerve grafts implanted into the site of complete thoracic spinal cord transection are permissive for the regeneration of intraspinal axons. Regeneration of supraspinal axons was detected only when grafts were inserted higher into the cervical cord rather than the thoracic cord (Richardson et al., 1984). Nevertheless, ascending and descending spinal axons regenerate into peripheral nerve grafts for up to 35 mm (Aguayo et al., 1981; David and Aguayo, 1981, 1985). Given the permissive nature of peripheral nerve grafts, numerous studies have sought to further enhance the regeneration of axons by combining the transplantation of peripheral nerves with additional treatment strategies such as the application of FGF (Lee et al., 2002, 2004, 2006, 2010), neurotrophins (Kobayashi et al., 1997; Oudega and Hagg, 1999; Yick et al., 1999; Hiebert et al., 2002; Storer et al., 2003; Feng et al., 2008), immunosuppression (Voda et al., 2005), elevation of intracellular cAMP (Han et al., 2004), and conditioning lesions (Chong et al., 1996; Han et al., 2004). These combination strategies result in
improved axon regeneration from numerous intraspinal and supraspinal neuron populations.

Transplantation: Extracellular matrix-like substrates

In 1979, Singer and colleagues proposed the “blueprint hypothesis of neuronal pathway patterning”, whereby axons grow through linearly aligned channels or spaces between the developing cellular terrain and ECM. Since then, the transplantation of ECM components and synthetic biomaterials has largely adhered to this hypothesis. This approach began with the transplantation of semi-fluid collagen into a complete transection injury into which catecholaminergic axons regenerate (de la Torre, 1982). The type I collagen gel, together with endogenous collagen, forms a permissive cross-linked lattice network with abundant interfibrillary spaces, and functions as a scaffold for the regeneration of intraspinal and supraspinal axons (de la Torre, 1982; Gelderd, 1990; Marchand and Woerly, 1990), including the sprouting of a few corticospinal axons (Joosten et al., 1995). Collagen tubes and fibers are permissive for axonal regeneration into and/or through lateral hemisections (Liu et al., 2001a, b; Li et al., 2009) and complete transection injuries (Spilker et al., 2001; Yoshii et al., 2003; 2004; 2009; Fukushima et al., 2008), respectively. When collagen is combined with growth factors, there is improved axonal regeneration (including corticospinal axons) as well as modest recovery of hindlimb locomotion (Houweling et al., 1998a, b; Joosten et al., 1999; Han et al., 2009).

In addition to collagen, other ECM matrices and biomaterials are permissive for axonal growth after SCI (reviewed by Zhang et al., 2005; Straley
et al., 2010). Fibronectin conduits allow for the regeneration of sensory axons across the injured dorsal root entry zone (DREZ; Priestly et al., 2002) and of most types of injured spinal axons (King et al., 2003). Hydrogels are permissive for the regeneration of supraspinal axons after complete transection (Woerly et al., 2001; Tsai et al., 2004), and this effect improves when hydrogels are combined with growth factors (Tsai et al., 2006). The polysaccharide, alginate, also allows for axons to regenerate across sites of complete transection (Suzuki et al., 2002; Kataoka et al., 2004; Prang et al., 2006). Injectable biomaterials may be especially useful because the scaffold assembles in vivo. Examples of injectable materials, besides collagen, that have achieved some axonal growth after SCI include hyaluronan methylcellulose (Shoichet et al., 2007), self-assembling nanofibers (Tysseling-Mattiace et al., 2009), and fibronectin and fibrin + fibronectin (King et al., 2010). Finally, biomatrices have become increasingly sophisticated and are now capable of gene delivery by coating with lipoplexes (De Laporte et al., 2009) or incorporating growth factors or binding motifs for specific surface receptors that regulate axonal growth (reviewed by Geller and Fawcett, 2002).

**Transplantation: Fibroblasts**

Skin fibroblasts are very abundant, highly prolific, and relatively easy to and transduce. Therefore, numerous groups use genetically modified fibroblasts as transplantation strategies to release factors that may enhance axon growth. When grafted into the uninjured spinal cord, fibroblasts that secrete NGF promote a robust ingrowth of sensory axons (Tuszynski et al., 1994). Fibroblasts
that release NT-3 are permissive for the growth of corticospinal axons when implanted into sites of dorsal spinal cord hemisection (Grill et al., 1997a). Furthermore, when grafted into sites of acute spinal cord contusion, fibroblasts modified to secrete various soluble growth factors increase the growth of supraspinal axons (McTigue et al., 1998; Liu et al., 1999; Blesch and Tuszynski et al., 2003). Such fibroblasts also improve axonal growth when implanted into sites of chronic contusion injury (Grill et al., 1997b; Jin et al., 2000a, 2002; Tuszynski et al., 2003). In addition, fibroblasts that release soluble growth factors enhance axon growth across regions of SCI that contain high levels of inhibitory molecules such as CSPGs (Jones et al., 2003). A common result of the studies mentioned above is that minimal axonal growth is observed when fibroblasts are not genetically modified. In contrast, Franzen et al. (1999), reports that transplantation of non-genetically modified meningeal fibroblasts may be permissive for the growth of supraspinal axons due their endogenous secretion of growth factors. This result is very surprising given the significant role meningeal fibroblasts play in formation of the glial scar.

**Transplantation: Schwann cells**

The clinically relevant autologous transplantation of SCs was first proposed by Bunge (1975) due to their permissive nature for axonal growth. In this approach, a peripheral nerve would be removed from the person with SCI and placed in culture to produce a population of SCs that would be implanted into the injured cord. The use of this strategy is supported by studies demonstrating that SCs express numerous soluble, cell surface, and ECM proteins that promote
axon regeneration, and that they also myelinate axons, provide neuroprotective effects, as well as prevent or fill cavities that form after contusive injury (reviewed by Bunge, 1994, 2001; Oudega and Xu, 2006). Blakemore (1977) first established that SCs can remyelinate CNS axons. Then, shortly after the seminal studies demonstrating CNS axon regeneration into grafts of peripheral nerve, Duncan et al. (1981) performed the first transplantation of SCs into demyelinated mouse spinal cord and confirmed xenogenic remyelination.

Numerous additional studies, using various experimental models, also confirm that the transplantation of SCs enhances axonal growth and myelination after SCI. For example, SCs transplanted into transection injuries of the thoracic spinal cord are permissive for local axonal regeneration (Wrathall et al., 1984) and regeneration of corticospinal axons below a thoracic lesion in the neonatal rat (Kuhlengel et al., 1990). SCs enclosed in rolls of collagen and transplanted into adult rats, promote axon regeneration as well as myelination analogous to peripheral nerve grafts (Paino and Bunge, 1991; Paino et al., 1994). Intraspinal axons growth into purified populations of SCs grafted into sites of thoracic compression (Martin et al., 1991, 1993) or dorsal hemisection injury (Montgomery et al., 1996). When SCs are transplanted into small cervical lesions of the corticospinal tract (CST), corticospinal axons sprout into the grafts (Li and Raisman, 1994). Lastly, when a preformed cord of SCs mixed with Matrigel (which contains collagen type IV and laminin) is transplanted into a complete thoracic spinal cord transection gap, intraspinal but not supraspinal axons regenerate into the graft (Xu et al., 1995a, 1997). Some supraspinal
axons regenerate, however, if pre-formed SC and Matrigel grafts in hemi-channels are implanted into a site of hemi-transection in the thoracic cord (Xu et al., 1999).

SCs are the primary substrate for combination strategies that further enhance the regeneration of both intraspinal and supraspinal axons after SCI (reviewed by Bunge, 2008; Silver, 2008; Fortun et al., 2009). The implantation of SCs together with an infusion of neurotrophins or other soluble growth factors improves axon regeneration (Xu et al., 1995b; Oudega et al., 1997; Bamber et al., 2001; Bregman et al., 2002; Iannotti et al., 2003; Meijs et al., 2004); this also occurs when SCs are genetically modified to secrete neurotrophins (Menei et al., 1998; Weidner et al., 1999a; Hurtado et al., 2006; Golden et al., 2007). Axon regeneration is increased with the combination of SCs transplantation and the administration of methyprednisolone (Chen et al., 1996; Guest et al., 1997a), an antibody raised against an axon growth inhibitor in myelin (Guest et al., 1997b), or chondroitinase (Chau et al., 2004; Fouad et al., 2005). In addition, elevation of intracellular cAMP enhances the ability of transplanted SCs to myelinate axons as well as promote axon growth, and this combination strategy substantially improves hindlimb locomotion (Pearse et al., 2004). SCs may be genetically modified to express polysialylated NCAM (PSA-NCAM) and this increases myelination and axon growth (Papastefanaki et al., 2007; Zhang et al., 2007a). Given the numerous benefits of SCs, some investigators engineer biodegradable scaffolds to enhance their survival and ability to regenerate axons after SCI (reviewed by Tabesh et al., 2009). Finally, SCs may be derived from precursors
in the skin, when the precursors are treated with appropriate factors. Biernaskie et al. (2007) demonstrates that these skin precursors promote axon growth, remyelination, and functional recovery after contusion injury, indicating another potential source for autologous transplantation; because of their enhanced migratory propensity and less astrogliosis after their transplantation, they may be more effective than adult peripheral nerve derived SCs.

**Transplantation: Embryonic and fetal tissue**

Given the ability of embryonic CNS tissue to repair itself after injury, numerous studies have sought to transplant embryonic or fetal tissue after SCI to promote both neuroprotection and axon regeneration. Scientists learned to transplant embryonic tissue more than a century ago but the benefits of such transplants for SCI have only become elucidated in the past 20-30 years. For example, fetal transplants are capable of rescuing supraspinal neurons from atrophy (Bregman and Reier, 1986). Such grafted tissue develops into CNS-like parenchyma composed of neurons, oligodendrocytes, and astrocytes (Bernstein and Goldberg, 1986). Many of the immature astrocytes appear to migrate out of the transplant and into the spinal cord (Goldberg and Bernstein, 1987) and grafted neurons project axons into the host spinal cord (Reier et al., 1986; Jakeman and Reier, 1991; Reier et al., 1992). In addition, embryonic transplants allow for intraspinal and supraspinal axon growth into the acute (Reier et al., 1986; Tessler et al., 1988; Jakeman and Reier, 1991) and chronic (Houlé and Reier, 1988; Akesson et al., 2001) injury sites as well as distal to the injury (Bamber et al., 1999). Axon growth into the graft is enhanced by the application
of neurotrophic factors (Bregman et al., 1997; Coumans et al., 2001) which results in the formation of some synapses as evident by electrophysiological activity upon stimulation of dorsal root fibers (Itoh et al., 1996) as well as functional recovery (Akesson et al., 2001; Zurita et al., 2001; Lynskey et al., 2006). Although CSPG expression does not appear to be altered by grafts of embryonic tissue (Lemons et al., 1999), an additional benefit of fetal transplants is the obliteration of cystic cavities and syringomyelia (Falci et al., 1997; Akesson et al., 2001; Forthofer and Wirth, 2001; Wirth et al., 2001; Guest et al., 2006).

**Transplantation: Progenitor cells**

In contrast to transplants of embryonic and fetal tissue, the transplantation of progenitor cells into sites of SCI does not typically result in the differentiation of new neurons, just as the endogenous population of progenitor cells in the spinal cord does not differentiate into neurons (Akiyama et al., 2001; Sasaki et al., 2001; Cao et al., 2001, 2002; Vroemen et al., 2003; Yan et al., 2004; Pallini et al., 2005). However, progenitor cells differentiate into neurons in vitro, strongly emphasizing the lesion environment is not permissive for neurodifferentiation. Therefore, intrinsic (i.e. basic helix-loop-helix transcription factors) and extrinsic strategies (i.e. retinoic acid or BDNF) are required prior to transplantation to prime the differentiation of progenitor cells into neurons in order to overcome environmental signals at the lesion site (McDonald et al., 1999; Ikeda et al., 2004; Setoguchi et al., 2004; Hofstetter et al., 2005; Hamada et al., 2006; Bonner et al., 2010).
Numerous types of progenitor cells transplanted after SCI may integrate with the host tissue (reviewed by Myckatyn et al., 2004; Kim et al., 2007; Nandoe-Tewarie et al., 2009). After transplantation into the injured spinal cord, many progenitor cell types differentiate into oligodendrocytes that successfully remyelinate demyelinated axons after transplantation (Keirstead et al., 1999; Akiyama et al., 2001; Sasaki et al., 2001). Progenitor cells also promote axon growth due to the secretion of growth factors (Lu et al., 2003; Xiao et al., 2005; Zhang et al., 2006), reduction in scar formation (Teng et al., 2002; Hill et al., 2004; Pallini et al., 2005) and provision of a permissive substrate and scaffold (Ankeny et al., 2004; Pfeifer et al., 2004; Davies et al., 2006). Progenitor cells that promote axonal growth when transplanted after SCI include bone marrow stromal cells (Ankeny et al., 2004; Parr et al., 2008), umbilical cord stem cells (Nishio et al., 2006), as well as adult and embryonic neural progenitor cells (Teng et al., 2002; Lu et al., 2003; Vroemen et al., 2003; Pfeifer et al., 2004; Pallini et al., 2005; Xiao et al., 2005, 2007; Boido et al., 2009; Olson et al., 2009). The benefits of transplanted progenitor cells may be, in part, due to their differentiation into immature glia, given that the direct transplantation of immature astrocytes, radial glia, O-2A progenitors, or tanycytes promotes axon growth from intraspinal and supraspinal neurons into the lesion site (Kliot et al., 1990; Olby and Blakemore, 1996; Joosten et al., 2004; Lee et al., 2005) as well as into the distal host spinal cord (Davies et al., 2006). However, the differentiation of progenitor cells into some types of glia may inhibit axonal growth. For example, the transplantation of type-1 astrocytes appears to reconstitute a glia limitans
around the injury site (reviewed by Blakemore, 1992). Therefore, strategies
designed to direct the differentiation of transplanted progenitor cells into specific
lineages are likely to improve axon regeneration and functional recovery.

Progenitor cells genetically modified to secrete growth factors further
enhance the growth of intraspinal and supraspinal axons after SCI (Lu et al.,
2005; Cao et al., 2005; Sasaki et al., 2009). However, CNTF appears to produce
both positive and negative effects. For example, blocking CNTF reduces scar
formation and promotes supraspinal axon growth after transplantation of neural
progenitors (Ishii et al., 2006; Davies et al., 2008), but remyelination by
oligodendrocyte progenitors may require CNTF (Cao et al., 2010). Nevertheless,
combinations of growth factors and progenitor cells are typically quite beneficial.
Perhaps the most striking example of this is the combination of bone marrow
stromal cell transplantation with conditioning lesions of the DRG and a gradient
of NT-3; this results in a robust regeneration of sensory axons into and out of
cervical dorsal column lesions (Kadoya et al., 2009). The transplantation of adult
neural progenitor cells together with the application of chondroitinase and the
infusion of EGF, FGF, and PDGF, also enhances the growth of supraspinal
axons (Karimi-Abdolrezaee et al., 2010).

**Transplantation: Olfactory ensheathing cells**

Olfactory ensheathing cells (OECs) are specialized cells that guide
olfactory receptor axons from nasal mucosa into the olfactory bulb of the CNS.
After transplantation they share characteristics of both SCs and astrocytes in
being able to myelinate CNS axons, secrete components of the basal lamina,
and also accompany the entry of axons into the CNS (reviewed by Ramon-Cueto and Valverde, 1995; Kocsis et al., 2009). When transplanted into the lesioned DREZ, OECs permit sensory axons from the DRG to growth into the spinal cord (Ramon-Cueto and Neito-Sampedro, 1994). The transplantation of OECs also promotes corticospinal axon growth through the lesion site as well as recovery of forelimb function after cervical hemisection (Li et al., 1997). Furthermore, long-distance axon regeneration of supraspinal axons is observed when OECs are transplanted at both the rostral and caudal interfaces following the complete transection and implantation of a SC bridge (Ramon-Cueto et al., 1998). In contrast, supraspinal axon regeneration is not observed when a similar paradigm is combined with a motor enrichment strategy (Moon et al., 2006a) and when OECs alone are transplanted after dorsal hemisection (Deumens et al., 2006a, b). Nevertheless, OECs transplanted four weeks after complete transection improve the regeneration of supraspinal axons through the lesion (Lu et al., 2002) and OEC-mediated axon regeneration is increased when combined with chondroitinase (Fouad et al., 2005; Vavrek et al., 2007).

**Intrinsic**

Despite numerous extrinsic treatment strategies for SCI, robust axon regeneration and recovery of function have not been observed, which has led to the dogma that the intrinsic state of the neuron also may need to be changed to a phenotype that is capable of axon regeneration. Accordingly, numerous intrinsic strategies have been developed and will be described briefly below, together with their rationale and potential mechanism of action.
Recreating the developmental phenotype

In 1928, Ramon y Cajal summarized differences between the regenerative phenotype of immature and mature CNS neurons using various injury models and transplantation experiments. Since then, many different types of immature CNS neurons are now well characterized according to their capacity for axonal regeneration (reviewed by Steeves et al., 1994; Nicholls and Saunders, 1996) after SCI (Kalil and Reh, 1979; Bregman and Goldberger, 1982; Carlstedt et al., 1987; Carlstedt, 1988), as well as upon transplantation into the injured and uninjured adult CNS (Houlé and Reier, 1988; Wictorin et al., 1992; Wictorin and Bjorklund, 1992; Li and Raisman, 1993; Davies et al., 1993, 1994; Blackmore and Letourneau, 2006a). Based on this work, “temporal windows” have been revealed for the development of different neuron populations. During this developmental time period, neurons of a given phenotype may grow neurites in vitro (Sagot et al., 1991; Dusrat et al., 1997; Borisoff et al., 2000; Goldberg et al., 2002) and regenerate their axons following either injury (Bernstein and Stelzner, 1983; Bregman et al., 1989; Kierstead et al., 1992; Hasan et al., 1993; Chen et al., 1995; Hafidi et al., 1999) or transplantation into the mature CNS (Fricker-Gates et al., 2002).

As the temporal windows have become elucidated for various neuron populations, the factors that mediate the observed changes in axon regeneration have been the focus of many studies. Some investigators suggest that a neuron’s temporal window may coincide with the onset of myelination, given that the duration of the window can be extended by the transplantation of immature
glia (Smith et al., 1986; Hafidi et al., 2004), embryonic tissue (Bregman et al., 1989; Bernstein-Goral and Bregman, 1993), suppression of myelination (Kierstead et al., 1992), application of chondroitinase (Hafidi et al., 2004), or inhibition of type IV collagen synthesis (Kawano et al., 2005). However, other investigators have reported that the inhibitory components in the scar, such as CSPGs (Bicknese et al., 1994) and myelin (Johnson et al., 1989; Li and Raisman, 1993; Bandtlow and Loschinger, 1997; Turnley and Bartlett, 1998), are permissive for axonal growth from embryonic CNS neurons. Furthermore, embryonic neurons appear to undergo a developmental switch in their response to myelin (DeBellard et al., 1996), that is regulated by endogenous cAMP levels (Cai et al., 2001), and occurs in the absence of myelin (Bouslama-Oueghlani et al., 2003). Thus, the expression of inhibitory components may be, at most, only partially responsible for mediating the temporal window. This is supported by observations from heterochronic co-cultures and transplantation experiments that reveal the extrinsic state of the CNS dictates some of the potential for axons to regenerate, but the intrinsic state of the maturing neuron is primarily responsible for the decline in regenerative capacity (Blackmore and Letourneau, 2006a). In accordance these experiments, numerous genes associated with axonal growth, including neurotrophin levels and their receptors, decline during development (Skene and Willard, 1981; Fitzgerald et al., 1991; reviewed by Jelsma and Aguayo, 1994). Given the relative importance of the intrinsic state for mediating the temporal window in development, recapitulating that state may be a useful strategy to enhance the regeneration of axons from mature neurons.
**Conditioning lesions**

The intrinsic state of mature neurons can be primed for regeneration. This is most prominent in a conditioning lesion paradigm, where some types of neurons demonstrate an enhanced capacity to regenerate 1-2 weeks after prior injury (McQuarrie et al., 1973, 1977, 1978; Forman et al., 1980; McQuarrie and Grafstein, 1981; Bisby, 1985; Cho and So, 1989). With respect to SCI, DRG neurons, which project axons into both CNS and PNS, can regenerate their central branches in the dorsal columns of the spinal cord only after the peripheral nerve branch has been cut earlier (Richardson and Issa, 1984; Chong, 1994; Smith and Skene, 1997; Neumann and Woolf, 1999). This effect also may occur when conditioning lesions are performed months after the initial injury (Ylera et al., 2009). Increased levels of intracellular cAMP (Neumann et al., 2002; Qui et al., 2002; Han et al., 2004) or the application of interleukin-6 (Cao et al., 2006) to DRGs are sufficient to mimic some, but not all, of the conditioning lesion-induced intrinsic changes in axon growth capacity. Conditioning lesions appear to overcome some axonal growth inhibitors such as CSPGs, but not semaphorins (Pasterkamp et al., 2001). Finally, conditioning lesions appear to enhance axon regeneration by inducing changes in gene expression and axonal transport (reviewed by Hoffman, 2010).

**Manipulations to neuronal somata**

When lesions are made proximal to the somata, greater axon regeneration is observed than when lesions are more distal (Richardson et al., 1984; So and Aguayo, 1985; Fernandes et al., 1999). This suggests instructive signals and/or
the amount of materials required for axonal growth may be greater between the growth cone and the somata when the distance is shorter. Although instructive signals from the growth cone are likely to be important, manipulations that target the somata alone are capable of enhancing axon regeneration. For example, inflammation near the somata of DRG neurons enhances the regeneration of their axons (Lu and Richardson, 1991). In addition, the implantation of a peripheral nerve graft into the vitreous, to provide diffusible signals to the somata (Berry et al., 1996, 1999), or lens injury (Leon et al., 2000) improves the regeneration of RGC axons. These effects may be, in part, mediated by the macrophage-derived calcium binding protein, oncomodulin (Yin et al., 2006).

With respect to SCI, the direct delivery of BDNF to the red nucleus increases the regeneration of rubrospinal axons (Kobayashi et al., 1997). Analogous to conditioning lesions, the effect of neurotrophins appears to be mediated by elevations in cAMP (Ming et al., 1997; Song et al., 1997; Cai et al., 1999; Li et al., 2003); the direct injection of a membrane permeable, synthetic analog for cAMP, dibutyryl-cAMP (db-cAMP) into the somata of brainstem neurons in the zebrafish enhances the regeneration of their axons in the spinal cord (Bhatt et al., 2004). Finally, the above mentioned studies demonstrate that manipulations of the somata induce an up-regulation of several genes known to regulate axon growth and are thus referred to as regeneration-associated genes (RAGs).

**Regeneration-associated genes**

Recognizing the ability of immature neurons to regenerate, Skene and Willard (1981) identified one of the first RAGs through experiments with
embryonic RGCs, and termed the gene, growth-associated protein 43 (GAP-43; Skene and Willard, 1981; reviewed by Skene, 1989). Since then, many additional RAGs have been identified and found to be transiently expressed in the somata of injured CNS neurons (Doster et al., 1991; Tetzlaff et al., 1991; Schmitt et al., 1999, 2003; Chaisuksunt et al., 2000; Carmel et al., 2001; Mason et al., 2002, 2003) as well as after conditioning lesions (Chong et al., 1996) or application of growth factors to the somata (Fournier and McKerracher, 1997; Fournier et al., 1997; Kobayashi et al., 1997; Storer et al., 2002, 2003). RAGs encode for many classes of proteins ranging from cytoskeletal elements to transcription factors (Muma et al., 1990; Funakoshi et al., 1993; Moskowitz and Oblinger, 1995; Frey et al., 2000; Bonilla et al., 2002; Cai et al., 2002; Tanabe et al., 2003; Veldman et al., 2007). Current studies are characterizing the regenerative phenotype by performing a large-scale analysis of gene expression patterns, using technology such as genetic or proteomic micro-arrays (reviewed by Wintzer et al., 2004) to reveal gene expression patterns in uninjured (Bonaventure et al., 2002; Gray et al., 2004; Arlotta et al., 2005) versus injured (Kury et al., 2004; De Biase et al., 2005; Di Giovanni et al., 2005a, b) CNS tissue. High content screening analysis of candidate genes from such studies may also confirm the function of novel RAGs in vitro (Moore et al., 2009; Blackmore et al., 2010). Importantly, the expression of individual RAGs is not sufficient to promote the regeneration of axons, but the expression of multiple RAGs may induce axon regeneration in the CNS (Bomze et al., 2001; Zhang et al., 2005).
**Second messengers**

Of the second messengers, cAMP appears to have the greatest influence on a neuron’s capacity to regenerate an axon. This was first demonstrated in the PNS, where Forskolin, an agonist for the cAMP synthetic enzyme adenylyl cyclase, increases the rate of axon regeneration (Kilmer and Calrsen, 1984). Forskolin or inhibition of an enzyme that degrades cAMP, phosphodiesterase (PDE), transforms growth cone lamellae into neurite endings (Forscher et al., 1987). Growth cones also turn towards the source of exogenously applied cAMP (Lohof et al., 1992), and many of the neurotrophin and axonal guidance signal transduction pathways are influenced by intracellular elevations in cAMP and its effector, protein kinase A (PKA; Ming et al., 1997; Song et al., 1997). Cultures of cerebellar and DRG neurons, grown on myelin, extend neurites upon exposure to either neurotrophins or db-CAMP, in a PKA dependent manner (Cai et al., 1999). Furthermore, axon growth from early postnatal corticospinal neurons may be blocked in vivo following the application of an inhibitor to PKA at the site of the lesion (Cai et al., 2001).

Given that cAMP levels mediate axonal growth, regulating the PDEs may serve as an efficient intrinsic treatment strategy to enhance axon regeneration. Experimental evidence that supports this includes a decrease in type-II PDE activity following the application of NGF to PC-12 cells (Bentley et al., 2001). In addition, the elongation of neurites from DRG neurons is dependent upon the inhibition of type-III PDE by cyclic guanosine mono-phosphate (cGMP; Tsukada et al., 2002). More importantly, inhibition of the type-IV PDE promotes significant
axon growth and functional recovery in the PNS (Walokonis and Poduslo, 1998) and after SCI (Nikulina et al., 2004; Pearse et al., 2004).

With respect to other second messengers, intracellular levels of calcium function indirectly through calmodulin to regulate adenylyl cyclase (reviewed by Ferguson and Storm, 2004). This may be one of the links whereby neuronal activity may induce axonal growth (Carmichael and Chesselet, 2002; Goldberg et al., 2002) and functional recovery after injury (reviewed by Fouad and Pearson, 2004). However, intracellular elevations in calcium activate protein kinase C (PKC) signaling (reviewed by Chakravarthy et al., 1999) and this effect may reduce axonal growth, discussed below. Noteworthy are the antagonistic roles that cAMP and cGMP typically have on axonogenesis (Shelly et al., 2010). This may be, in part, due to an association between intracellular cGMP levels and activation of the Rho subfamily of GTPases, which are well known to regulate axonal growth.

**Rho/Rock**

The cornerstone of motility-based signaling pathways is the Rho subfamily of monomeric GTPases, whose members include RhoA, Rac, as well as Cdc42. These enzymes function as molecular switches that are active when bound to guanosine tri-phosphate and inactive when bound to guanosine di-phosphate. Their function is further regulated by activator proteins and exchange factors. In neuronal cell lines RhoA stimulates the formation of bundles of actin and myosin, termed stress fibers, which attach to focal adhesion complexes in the membrane, while Rac reorganizes actin into lamellipodia and Cdc42 induces the formation of
filopodia (Nobes and Hall, 1995). In this way, the activation of Rac1 and Cdc42 promotes neurite outgrowth and RhoA mediates growth cone collapse (Jalink et al., 1994; Kozma 1997).

The most well characterized downstream factor for Cdc42 and Rac signaling in the growth cone is p21-activated kinase (PAK; Manser et al., 1994; reviewed by Bagrodia and Cerione, 1999). Activated PAK appears to have antagonistic effects on growth cone cytoskeletal-dynamics. On one hand, activated PAK phosphorylates LIM kinase (Edwards et al., 1999; Sumi et al., 2001a), which then phosphorylates and inactivates cofilins to promote growth cone collapse (reviewed by Gungabissoon and Bamburg, 2003). However, PAK also phosphorylates and thereby inactivates myosin light chain kinase (Sanders et al., 1999). This prevents the phosphorylation and activation of myosin light chain (MLC), and thus inhibits the generation of force and stress fibers by myosin II which cause growth cone collapse (Sanders et al., 1999; reviewed by Burridge, 1999; Brown and Bridgman, 2004). The signaling pathways regulated by PAK also are regulated by one of the major downstream effectors of RhoA, the serine/threonine Rho-associated kinase (ROCK; Leung et al., 1995a, b; Matsuri et al., 1996). ROCK mediates growth cone collapse and retraction through collapsing mediator protein-2 (Arimura et al., 2000), as well as through the activation of LIM kinase and cofilins (Maekawa et al., 1999; Ohashi et al., 2000; Sumi et al., 2001b). Finally, ROCK antagonizes PAK by activating MLC, which leads to increased contractility, stress fiber formation, and growth cone collapse (Kimura et al., 1996; reviewed by Burridge, 1999; Brown and Bridgman, 2004).
The intrinsic effects that cAMP has on axonal growth (discussed above) have also been linked to Rho/ROCK signaling. For example, PKA reduces the activation of MLC (Conti and Adelstein, 1981). This leads to the rapid reorganization of actin filaments in the lamellipodia and stress fibers that is required for the advance of microtubules and organelles into the distal portion of the growth cone during process elongation in neurons (Forsher et al., 1987) and astrocytes (Baorto et al., 1992). PKA also directly inactivates RhoA to induce neurite formation (Tigyi et al., 1996; Dong et al., 1998; Kreibich et al., 2004), and indirectly inactivates RhoA through the phosphorylation of the p75 receptor (Higuchi et al., 2003). In addition, PKA induces neurite outgrowth through the activation of Rac and Cdc42 (Chen et al., 2003; reviewed by Bandtlow, 2003).

There is a convergence of signaling onto RhoA for the semaphorins (reviewed by Negishi et al., 2005), the ephrins (reviewed by Goldshmit et al., 2006), as well as the CSPGs and the inhibitory factors found in myelin. This signaling is in part mediated by the low-affinity neurotrophin receptor, p75, which lacks intrinsic enzymatic activity, but in combination with NgR activates RhoA (Yamashita et al., 1999, 2002; Wang et al., 2002b; Wong et al., 2002; Monnier et al., 2003; Schweigreiter et al., 2004). RhoA inhibitors are capable of promoting axonal growth across myelin and CSPGs following optic nerve or SCI (Lehmann et al., 1999, Dergham et al., 2002; Niederost et al., 2002; Jain et al., 2004). Thus, several groups have focused on Rho/Rock signaling as an intrinsic strategy to enhance axon regeneration and functional recovery in vivo (reviewed by Mueller et al., 2005; Kubo and Yamashita, 2007, Kubo et al., 2007, 2008).
Both Rho and ROCK antagonists block neurite outgrowth inhibition by myelin and MAG (Lehmann et al., 1999; Dergman et al., 2002; Fournier et al., 2003), promote axon growth after optic nerve injury (Lehmann et al., 1999; Bertrand et al., 2005) and enhance axon growth as well as functional recovery after SCI (Dergham et al., 2002; Fournier et al., 2003; Wu et al., 2009). Currently, the Rho antagonist, Cethrin, is being tested clinically (reviewed by Fehlings and Baptiste, 2005; Kwon et al., 2010).

**Receptor tyrosine kinases**

The receptor tyrosine kinases include a large variety of surface membrane proteins that are well recognized to regulate neurite outgrowth. There are several classes of tyrosine kinase receptors, including the neurotrophin receptors (Trks; reviewed by Boyd and Gordon, 2003; Huang and Reichardt, 2003) and ephrins/Eph receptors (reviewed by Goldshmidt et al., 2006). The receptor tyrosine kinases also are regulated by PTPs (reviewed by Enssljen-Craig and Brady-Kalnay, 2004) and in particular PTP-σ, which is a receptor for CSPGs that inhibits neurite growth (Shen et al., 2009). Together, the tyrosine kinase receptors and the PTPs regulate numerous converging and diverging signaling pathways that mediate axonal growth, including Ras/Erk, Phospholipase C (PLC), and phosphatidylinositol-3 kinase (PI3K), and Rho/ROCK.

Intrinsic strategies that utilize receptor tyrosine kinase signaling pathways to promote axon regeneration include the application of neurotrophins to the somata (Fournier and McKerracher, 1997; Fournier et al., 1997, Kobyashi et al., 1997) as well as gene therapy and pharmacological approaches. For example,
lentiviral-mediated expression of TrkB in cortical projection neurons activates Erk and enhances the growth of their axons after subcortical injury when combined with infusion of BDNF into the lesion (Hollis et al., 2009). In addition, Miura et al. (2000) reported that adenovirus-mediated expression of a constitutively active member of the Ras/Erk pathway activated Erk in axons and induced their regeneration after SCI. Pharmacological treatments also seek to activate these pathways to promote axon regeneration (Gold and Zhong, 2004; Uwabe et al., 2006). Chemical screens to identify novel compounds (Usher et al., 2010) may elucidate new pharmacological strategies. Finally, elevations in intracellular cAMP may mediate effects on axon regeneration via receptor tyrosine signaling pathways by PKA and Epac1 activation of the Ras/Erk pathway (Vossler et al., 1997; de Rooij et al., 1998; Sidovar et al., 2000; Christensen et al., 2003) and PLC (Kim et al., 1989; Schmidt et al., 2001; Song et al., 2002).

**Integrins and cell adhesion molecules**

The integrins, a ubiquitous family of heterodimeric receptors composed of both alpha and beta subunits, regulate a variety of signaling cascades, including the Rho/Rock and Ras/Erk pathways (reviewed by Clegg, 2000; DeMali et al., 2003). Depending on the extracellular context and their recycling at the growth cone, integrins may mediate axon growth (Condic and Letourneau, 1997; Blackmore and Letourneau, 2006b; reviewed by Lemons and Condic 2008) or growth cone collapse (Lemons and Condic, 2006). Immature neurons regulate their integrin expression to rapidly adapt their ability to grow axons in the presence of inhibitory proteoglycans (Condic et al., 1999). This effect can be
restored in adult neurons following the adenoviral-mediated expression of α-integrin as an intrinsic treatment strategy (Condic, 2001).

The CAMs are dynamically regulated during axonal growth and mediate several intracellular signaling cascades (reviewed by Kamiguchi and Lemmon, 1997; Long and Lemmon, 2000; Kiryushko et al., 2004). Since the intracellular domains of CAMs do not contain catalytic activity, neurite outgrowth is at least partially dependent on their association with and activity of the FGF receptor (Williams et al., 1994). Similar to the receptor tyrosine kinases discussed above, neurite outgrowth induced by CAM-dependent FGF receptor signaling is mediated by the Ras/Erk cascade (Perron and Bixby, 1999; Dimitropoulou and Bixby, 2000), as well as the PLCγ/PKC cascade (Bixby, 1989; Bixby and Jhabvala, 1990; Kolkova et al., 2000). With respect to intrinsic treatment strategies, L1 expression is required for brainstem axon regeneration in zebrafish (Becker et al., 2004), and AAV-mediated expression of the CAM, L1, in the injured spinal cord promotes axonal growth from supraspinal neurons and functional recovery (Chen et al., 2007). Although CAMs promote axonal growth, the activation of PKC may be a downstream effector for myelin- and CSPG-mediated inhibition of axonal growth after SCI (Sivasankaran et al., 2004). Thus, the effects of CAMs appear to be context dependent.

**Axonal transport and translation**

In order to induce axon regeneration, signals are transported from the site of injury to the somata and molecules synthesized in the somata are delivered to the growth cone. Proteins and complexes that mediate axonal growth through
retrograde transport include c-Jun terminal kinase (JNK; Cavalli et al., 2005) and neurotrophins bound to their receptors (reviewed by Hirokawa and Takemura, 2004). During axonal growth numerous proteins and mRNAs are transported anterogradely, and selective impairment of slow anterograde axonal transport has been implicated in axon regeneration failure (McKerracher et al., 1990a, b; reviewed by Hoffman, 2010). Local growth cone translation appears necessary for growth cone formation, axonal guidance, and neuronal survival, but not for elongation of axons (Campbell and Holt, 2001; Brittis et al., 2002; Li et al., 2004; Verma et al., 2005; Blackmore and Letourneau, 2007; Cox et al., 2008). The regulation of protein synthesis by phosphatase and tensin homolog (PTEN) in both the soma and growth cone has been identified as a key inhibitor of axonal growth and this signaling pathway is currently a major target for intrinsic strategies (reviewed by Park et al., 2010).

PTEN antagonizes PI3K receptor tyrosine kinase signaling. This has two primary effects, the regulation of new protein synthesis through inhibition of mTOR and the activation of glycogen synthesis kinase (GSK)-3β. PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) in the membrane. This is required for neurotrophin-induced neurite outgrowth (Kimura et al., 1994; Jackson et al., 1996) through the inhibition of GSK-3β (Cross et al., 1995). PTEN and GSK-3β also are activated by semaphorin signaling (Eickholt et al., 2002) and mediate growth cone collapse (Chadborn et al., 2006). With respect to new protein synthesis, mTOR regulates 5' cap-dependent translation (reviewed by Ma and Blenis, 2009). Importantly, the deletion of PTEN
dramatically enhances RGC (Park et al., 2008) as well as corticospinal axon regeneration (Liu et al., 2010), and this is likely accomplished through modified axonal growth signaling as well as increased protein translation.

**Transcription factors: Immediate early genes**

Gene transcription is required for axon elongation (Tanabe and Jessell, 1996) and regeneration (Smith and Skene, 1997). After axonal injury there is a transient increase in the expression of many RAGs and this is mediated, in part, by the injury-induced expression of immediate early genes (IEGs) (reviewed by Herdegen and Leah, 1998). IEGs, such as Fos and Jun proteins, homodimerize and heterodimerize to make various activator protein (AP)-1 transcription factors. Of these components, c-Jun expression increases in both intraspinal and supraspinal neurons after injury (Houlé et al., 1998; Broude et al., 1999; Jin et al., 2000b; Raivich et al., 2004), and c-Jun is then activated by the retrograde translocation of JNK (reviewed by Verhey and Rapoport, 2001); this expression and activation is required for axonal outgrowth (Lindwall et al., 2004; Raivich et al., 2004). In addition to making the AP-1 family of transcription factors with Fos, Jun proteins heterodimerize with other transcription factors such as members of the activating transcription factor (ATF) family (reviewed by Hai and Hartman, 2001). Analogous to conditioning lesions, ATF-3 is expressed in DRGs and motor neurons after peripheral nerve injury (Tsujino et al., 2000; Averill et al., 2004; Stam et al., 2007), but not after injury to the proximal branch of DRGs (Seiffers et al., 2006). ATF-3 is also expressed in CNS neurons that regenerate into peripheral nerve grafts (Campbell et al., 2005). Furthermore, adenoviral-
mediated expression of ATF-3 induces neurite elongation as well as prevents neuronal death (Nakagomi et al., 2003), and overexpression in DRGs increases the regeneration of their axons and expression of RAGs (Seijffers et al., 2007). Together, these studies demonstrate that IEGs mediate injury-induced transient cascades of gene expression, and suggest that in areas of the CNS where IEGs are expressed after injury, the chromatin for some RAGs is, at least, partially open for transcription factors to bind.

**Transcription factors: CREB**

The basic-leucine zipper transcription factor, cAMP response element binding protein (CREB), is a member of the ATF family of transcription factors and the phosphorylation of CREB ser133 is the most direct way cAMP and PKA induce changes in transcription (Gonzalez and Montminy, 1989). Phosphorylation of CREB allows for dimerization with other members of the ATF family of transcription factors that together bind to cAMP response elements (CREs). Phospho-CREB is associated with neurons that have GAP-43 positive, actively growing axons (Schmidt et al., 1998), and therefore through binding to cAMP response elements (CREs) in cis-regulatory regions, CREB induces the expression of RAGs as part of a feed-forward transcriptional regulatory loop (MacGillavry et al., 2009). In this way, NGF- and NCAM-induced neurite outgrowth are likely dependent upon CREB (Jessen et al., 2001; Cheng et al., 2002). Expression of a dominant-negative CREB blocks the ability of BDNF and db-cAMP to overcome axon growth inhibition by myelin and constitutively-active
CREB is sufficient to promote modest growth of lesioned dorsal column axons (Gao et al., 2004).

**Transcription factors: Krüppel-like factors**

The Krüppel-like factor (KLF) family of zinc finger transcription factors are important intrinsic regulators of axonal growth and states of differentiation (Nandan and Yang, 2009). The KLFs belong to the relatively large family of serum protein (SP)-1-like transcription factors and bind to CACCC and/or G/C rich motifs in cis-regulatory regions. KLF6 expression is induced in the mammalian DRG following sciatic nerve transection (Nilsson et al., 2005). KLF7 is important to axonal growth in the CNS (Laub et al., 2005), and induces expression of TrkA (Lei et al., 2005), L1, and GAP-43 (Kajimura et al., 2007).

Comparison of microarray data from RGCs with injured or uninjured axons reveals increases in the expression of KLF6 and KLF7 in regenerating zebrafish neurons (Veldman et al., 2007). Using mouse RGCs, Moore, et al. (2009) report similar microarray results, where KLF6 and KLF7 expression increases during the temporal window in development when RGCs are capable of axon regeneration. In addition, their analyses find KLF4 and KLF9 to increase in expression as the RGCs mature and the temporal window “closes”. In vitro, they find expression of KLF4 and KLF9 to inhibit neurite growth and the repression of these inhibitors appears to be more potent than the ability of KLF6 and KLF7 to promote neurite growth. Finally, they demonstrate that young postnatal animals, in which KLF4 is knocked-out, are capable of re-growing some RGC axons after crush injury. Thus, KLF family members, in part, regulate the regenerative
phenotype of CNS neurons and their manipulation may be an important component of intrinsic treatment strategies.

**Transcription factors: basic helix-loop-helix**

Members of the basic helix-loop-helix (bHLH) family of transcriptional regulatory proteins are key components in a wide array of developmental processes, with over 240 different members identified so far (reviewed by Murre et al., 1994; Massari and Murre, 2000). These proteins homodimerize and heterodimerize to mediate binding to the core hexanucleotide sequence, CANNTG, termed the E-box motif in cis-regulatory regions of the genome. The different members are primarily subdivided into six classes. Class I bHLH proteins, also known as the E proteins, are ubiquitously expressed and serve as the binding partners to the other classes. Class II bHLH proteins are of primary interest given their tissue-specific patterns of expression and role in neural determination and differentiation; this class includes members such as mammalian achaete-scute homolog-1 (MASH-1) and NeuroD (McLellan et al., 2002). In addition, most members of Class II do not heterodimerize and require the expression and availability of Class I proteins in order to bind E-box motifs. Members of Class III include the Myc family of transcription factors which mediate chromatin remodeling and have been implicated in numerous processes including oncogenesis (Frank et al., 2001; Guccione et al., 2006; reviewed by Amati et al., 2001), while members of Class IV include Mad and Max which regulate cell survival and cell death. Class V proteins are important regulatory factors in that they do not bind deoxyribonucleic acid (DNA), but rather sequester
the other bHLH factors through heterodimerization to limit their function. Thus, these proteins are referred to as inhibitors of differentiation (Id) proteins. Class VI proteins are also important regulators of differentiation, which includes Hairy and Enhancer of split (HES) proteins. The HES proteins mediate transcriptional repression and thus counteract the determination and differentiation functions of the Class II proteins.

During the development of the CNS, many class II bHLH proteins are transiently expressed and considered as pro-neuronal factors, in that their expression in progenitor cells induces determination and differentiation of numerous neuronal phenotypes, in a context dependent manner (reviewed by Guillemot, 1999; Powell and Jarman, 2008). For example, in vivo studies using retroviral vectors to over-express MASH-1 in the postnatal retina and cortex demonstrate that MASH-1 promotes a neuronal fate over a glial one (Cai et al., 2000). Due to their induction of pan-neuronal phenotypes, such as axonal growth, Class II bHLH factors are good candidates for intrinsic treatments to promote axon regeneration. In support of this, knock down of a class II protein, bHLHb3, recently identified in a microarray analysis of genes that may mediate the conditioning lesion effect of DRG neurons, reduces neurite outgrowth from F11 cells (Stam et al., 2007; MacGillavry et al., 2009). However, given the cell-type specific nature of Class II bHLH transcription factors, their use in intrinsic treatment strategies to induce axon regeneration will likely be dependent upon the phenotype of the neuron.
One of the most well characterized Class II bHLH proteins, MASH-1, is transiently expressed in subsets of neural progenitor cells as they depart the mitotic cycle, including but not limited to, those of the noradrenergic lineage (Johnson et al., 1990). Importantly, the noradrenergic and serotonergic brainstem neurons, as well as neuronal populations from other regions of the CNS, are completely abolished in MASH-1 knock-out mutants (Guillemont et al., 1993; Hirsch et al., 1998; Pattyn et al., 2004). Forced expression of MASH-1 in cultures of neural crest progenitors induces noradrenergic differentiation as well as up-regulated the expression of a RAG, the c-ret receptor for glial cell-line derived neurotrophic factor (GDNF; Lo et al., 1998; Dauger et al., 2001; Lo et al., 2002). The expression of c-ret is important given the requirement of GDNF for neurite outgrowth from noradrenergic neurons (Reiriz et al., 2002). Transfection of MASH-1 also increases the expression of RAGs such as NCAM and type III beta tubulin in neural ectoderm (Ferreiro et al., 1994) and embryonic progenitor cells (Ikeda et al., 2004). Microarray analysis of a neuroendocrine cell line in which MASH-1 expression is knocked down via RNAi, reveals the cis-regulatory region of several RAGs, such as adenylyl cyclase-9 and NDRG4, to contain E-box motifs regulated by MASH-1 (Ohki et al., 2002; Hu et al., 2004). MASH-1 also induces the expression of RAGs such as SCG10 (Mason et al., 2002) and Hu during differentiation of noradrenergic neurons (Stanke et al., 2004). MASH-1 expression in embryonic progenitor cells yields purified spinal motor neuron precursors that lack the expression of the axonal growth inhibitory receptor NgR, and promote functional recovery upon transplantation after SCI (Hamada et al.,
Furthermore, the expression of MASH-1 in cultures of embryonic hippocampal neurons and forebrain progenitors promotes neurite outgrowth (Castella et al., 1999; Torii et al., 1999) and, in P19 embryonal carcinoma cells, MASH-1 expression mediates their differentiation into functional neurons with axons, dendrites, and action potentials (Johnson et al., 1992; Farah et al., 2000). Although neurite outgrowth and the expression of GAP-43 is transiently maintained as endogenous MASH-1 expression decreases, forced expression does not interfere with differentiation (Soderholm et al., 1999). Finally, MASH-1 expression overrides local mechanisms in the telencephalon as well as the spinal cord and re-specifies dorsal/ventral identities (Fode et al., 2000; Parras et al., 2002). Together, these studies show that MASH-1 is a master regulator of determination as well as differentiation for several neuronal cell-types and is capable of inducing the expression of RAGs.

Additional class II bHLH factors, such as the NeuroD sub-family members, are expressed downstream of MASH-1, mediate the expression of RAGs such as GAP-43 and TrkB (Farah et al., 2000; Pleasure et al., 2000; Cau et al., 2002; Ito et al., 2003; Liu et al., 2004), and are required for terminal differentiation of numerous neuronal cell types (Ohtsuka et al., 1998). The NeuroD cis-regulatory region contains numerous E-box domains (Miyachi et al., 1999), and NeuroD expression during the differentiation of olfactory neurons and hippocampal granule cells requires the previous expression of MASH-1 (Pleasure et al., 2000; Cau et al., 2002). In vivo expression of MASH-1 in peripheral nerve also induces NeuroD expression (Perez et al., 1999) and a
cascade of class II bHLH protein expression in olfactory progenitor cells. NeuroD subfamily members as well as other class II bHLH proteins bind to cis-regulatory regions for GAP-43 and promote neurite outgrowth in various cell lines in vitro (Chiaramello et al., 1996; Kinney et al., 1996; McCormick et al., 1996; Noma et al., 1999; Cho et al., 2001; Uittenbogaard and Chiaramello, 2002, 2004; Uittenbogaard et al., 2003). Therefore, the expression of MASH-1 mediates a cascade of events that may change the phenotype of mature neurons to one that is capable of axon regeneration.

Several prominent studies demonstrate that the forced expression of class II bHLH family members, such as MASH-1, is capable of changing the phenotype of mature somatic cells. For example, expression of the bHLH factor MATH-1 generates new cochlear hair cells from both postnatal and adult non-sensory cells in vivo (Zheng and Gao, 2000; Kawamoto et al., 2003). In addition, the expression of NeuroD in the livers of diabetic mice promotes islet cell genesis and differentiation as evident by an increase in the expression of pro-insulin processing enzymes and other islet-specific transcripts (Kojima et al., 2003). Finally, the expression of MASH-1 alone trans-differentiates adult fibroblasts into neuronal phenotypes in vitro (Vierbuchen et al., 2010).

The class V bHLH proteins, the Id proteins, are important mediators of the bHLH transcription factors and their expression is increased in neurons following SCI (Kabos et al., 2002). Interestingly, Id proteins are implicated in axonal growth through the anaphase-promoting complex (APC), which degrades Id2 upon exit from the cell cycle, and the initiation of axonogenesis (Konishi et al.,
2004; Lasorella et al., 2006). Given that the Id proteins also function to sequester the Class I bHLH proteins from Class II bHLH proteins such as MASH-1; without having a Class I binding partner, the Class II proteins are then ubiquitinated and degraded (Ghil et al., 2002; Sriuranpong et al., 2002; Vinals et al., 2004; Vosper et al., 2007). Thus, in a progenitor cell’s final mitotic cycle, APC may promote axonogenesis by reducing Id2 levels so that pro-differentiation factors, such as MASH-1, may be able to initiate a feed-forward expression pattern. In addition, APC induced axonogenesis may be linked to KLF7 mediated expression of the cell cycle regulator p21 (Laub et al., 2005). Together, these studies suggest that the negative regulators of the bHLH family are likely to have strong effects on axon regeneration, analogous to those of the KLF family.

**Transcription factors: Retinoic acid receptors**

Numerous steroid hormones also regulate neuronal differentiation, but in a more regional manner, rather than exhibiting specificity for given neuronal subtypes. One of the most classic hormones is retinoic acid (RA), a vitamin A derivative involved in numerous aspects of differentiation in the CNS through the formation of numerous homodimer and heterodimer receptors that function as transcription factors (reviewed by Maden, 2001). Of particular interest, RA acts as a transcriptional activator of class II bHLH family members, such as MASH-1, at various stages in neuronal differentiation (Corral et al., 2003; Novitch et al., 2003; Kim et al., 2004; Elmi et al., 2007), including the formation of axonal projection patterns (Solomin et al., 1998; Sockanathan et al., 2003). RA may
elicit neurite outgrowth without the addition of exogenous growth factors (Rodriguez-Tebar and Rohrer, 1991; Corcoran and Maden, 1999; Middlemas et al., 1999), and induces the expression of RAGs such as the neurotrophin receptors when applied at specific concentrations (Haskell et al., 1987; Kaplan et al., 1993; Lucarelli et al., 1995). Lentiviral-mediated expression of the retinoic acid receptor (RAR)-β2, in corticospinal neurons enhances the growth of their axons after injury to the cervical CST (Yip et al., 2006); expression in the DRG enhances the growth of sensory axons in the spinal cord (Wong et al., 2006). Furthermore, neuronal cultures, from regions of the adult CNS where the RAR-β2 transcription factor is expressed, grow neurites on the inhibitory components of myelin when provided with a RAR-β2 agonist in vitro (Agudo et al., 2010).

**Epigenetic factors**

Epigenetic factors have a pivotal role on the regulation of gene expression patterns through their modification of chromatin. Such mechanisms include DNA methylation of enhancer and promoter regions as well as the acetylation of histones; the enzymes which mediate these methylation and acetylation states are often cofactors of various complexes of transcription factors (reviewed by Herman and Baylin, 2003). Perhaps the most prominent of these is the CREB binding protein (CBP/p300; Kwok et al., 1994). CBP/p300 binds to components of the basal transcriptional machinery and also functions as a protein bridge through binding to numerous transcription factors, including the IEGs, c-Jun and c-Fos (Bannister et al., 1995; Swope et al., 1996), KLF family members (Geiman et al., 2000), as well as bHLH proteins such as c-Myc and NeuroD (Kaneto et al.,
Through these transcriptional complexes, CBP/p300 attracts chromatin modifying enzymes, such as histone acetyltransferase, to expose transcriptionally silent regions (reviewed by Chan and La Thangue, 2004). Furthermore, since CBP is present in limiting amounts in the nucleus, competition for CBP between CREB and other factors provides another mechanism for transcriptional regulation (Delgado, 2002; Takahashi et al., 2002). Since a given transcriptional complex functions through binding to specific combinations of cis-regulatory elements, they are often considered as nodes for phenotypic patterns of gene expression. Thus, re-creating such transcriptional nodes may be an efficient way to modify the intrinsic state of adult neurons and create a phenotype that is more capable of axon regeneration.

**Differentiation of noradrenergic brainstem neurons and their role in locomotion**

The anatomy and development of supraspinal projections, including the noradrenergic population has been reviewed extensively (Lakke et al., 1997). Noradrenergic axons are unmyelinated and project throughout the entire spinal cord. Therefore the complete transection model of the thoracic spinal cord is the most efficient way to assure the entire population is injured and any observed increase in axonal growth is due to regeneration. Of the various axonal populations that are lesioned after complete transection, the noradrenergic neurons in the brainstem are excellent candidates to study transcription factors that mediate axon regeneration. This is due to their phenotypic expression of dopamine-beta hydroxylase (DBH), which not only allows for easy identification
of their axons, but also is used as a reporter to identify factors that regulate noradrenergic development.

The use of DBH as a reporter has elucidated several important factors, in addition to MASH-1, that mediate noradrenergic development. For example, the paired homeobox transcription factor, Phox2b, is transiently expressed in noradrenergic neurons during development, downstream of MASH-1 (Hirsch et al., 1998). This factor regulates axonal projection patterns (Pujol et al., 2000) and, analogous to MASH-1, Phox2b knock-out mice lack noradrenergic neurons of the locus coeruleus (Pattyn et al., 1999). Phox2b also binds to cis-regulatory elements for and increases the expression of RAGs such as NCAM (Valarche et al., 1993). In addition, forced expression of Phox2b in cultures of neural crest progenitors induces the expression of c-ret (Stanke et al., 1999) and in vivo mediates ectopic noradrenergic differentiation (Stanke et al., 1999; Dubreuil et al., 2000) as well as the expression of synaptic vesicle associated proteins (Patzke et al., 2001). Thus, both Phox2b and MASH-1 promote cascades of events leading to noradrenergic differentiation, including axonal outgrowth and synapse formation (Pattyn et al., 2000; Brunet and Pattyn, 2002). Although Phox2b promotes phenotype specific differentiation, Phox2b also synchronizes pan-neuronal phenotypes by inducing the up-regulation of pro-neuronal bHLH factors, MASH-1 and NGN2, and repressing the expression of the negative regulators HES5 and Id2 (Dubreuil et al., 2002). Finally, although MASH-1 can mediate neuronal differentiation in the absence of RA (Farah et al. 2000), RA is required for hindbrain differentiation of noradrenergic neurons, and exogenously
applied RA can produce ectopic noradrenergic neurons in vivo (Holzschuh et al., 2003).

Supraspinal noradrenergic neurons mediate a variety of functions. These functions include those of the autonomic nervous system and locomotion. Despite the clinical importance of regaining autonomic function after SCI, the majority of behavioral assessments in experimental models of SCI are based on the recovery of locomotion. The neural bases of locomotion in vertebrates have been extensively reviewed (Schmidt and Jordan, 2000; Grillner et al., 2008). The cortex as well as the basal ganglia are responsible for the initiation of goal-directed movement and determine which motor programs are active. However, given the scope of this dissertation and the behavioral observations reported in subsequent chapters, only the central pattern generators (CPGs) and brainstem command centers that mediate locomotion shall be introduced. Then, specific functions that supraspinal noradrenergic neurons may serve will be identified.

The motor pattern of locomotion is generated at the spinal level by “half-centre” CPGs, which function as a movement-related sensory feedback system initially described by Brown (1911). In this way, different extensor and flexor motor neuron/muscle groups are sequentially activated to produce a given movement, such as hindlimb locomotion. Whereas CPGs vary in their complexity, the major components typically consist of excitatory glutamatergic and inhibitory glycinergic interneurons, as well as the motor neurons, partitioned into ipsilateral and contralateral sides. The excitatory interneurons produce
individual bursts of activity onto ipsilateral motor neurons and inhibitory interneurons, as well as contralateral excitatory and inhibitory interneurons. The activity levels of the spinal CPGs are regulated by the brainstem command centers (reviewed by Dietz, 2003). In particular, glutamatergic afferents from the reticulospinal system integrate vestibular, visual, and other sensory information to regulate steering, posture, and locomotion. The mesencephalic locomotor region (MLR) together with the diencephalic locomotor region is responsible for initiating a given motor command that originates from the basal ganglia and cortex. The MLR is located at the mesopontine border, and similar to other brainstem command centers, at rest the MLR is inhibited by high levels of GABAergic activity from the basal ganglia. Upon activation, the MLR sends bilateral cholinergic and glutamatergic efferents to the reticulospinal system. The direct control of CPG activity by the reticulospinal glutamatergic system is then modified by both serotonergic raphespinal and noradrenergic coeruleospinal systems. Given that these axonal populations are unmyelinated and therefore slow conducting, they are thought to prime the CPGs for the fast glutamatergic afferents. Thus, the regeneration of noradrenergic axons after spinal cord injury may function to prime the CPG for activity.
CHAPTER 2

THE USE OF AN ADENO-ASSOCIATED VIRAL VECTOR TO ASSESS THE REGENERATION OF BRAINSTEM AXONS IN THE SPINAL CORD AFTER INJURY
Overview

Adeno-associated virus serotype II (AAV) was utilized to anterogradely trace brainstem axons in the spinal cord through the expression of green fluorescent protein (GFP) in their somata. AAV-GFP vectors were stereotaxically injected into the brainstem between 2 and 6 weeks before complete transection of the thoracic spinal cord and implantation of a Schwann cell (SC) bridge. GFP was only expressed in NeuN-positive brainstem neurons, including those of the noradrenergic population. The continual expression of GFP enabled easy visualization of brainstem axons that regenerated into the bridge. The numbers of GFP-labeled axons rostral to the bridge directly correlated with those of GFP-labeled axons in the bridge. The percentages of GFP-labeled axons that regenerated into the proximal bridge were similar in all animals. However, those with a greater axonal number rostral to the bridge (>4,900 axons/mm²) exhibited a five-fold increase in the percentage of axons found near the distal end of the bridge compared to animals with a lesser number (<4,900 axons/mm²). This suggests that GFP is not uniformly distributed throughout the axon but accumulates in distal regions with time. In addition, GFP-labeled axon-like structures were found in the caudal spinal cord beyond the bridge; these structures were present two days after injury and, therefore, were not regenerating axons. In sum, AAV-GFP infected neurons in the brainstem, and numerous axons were anterogradely labeled rostral to the site of spinal cord transection as well as those regenerating into the SC bridge.
**Background**

The histological assessment of axon regeneration from distinct neuronal populations is often achieved through targeted neuronal tracing techniques. Anterograde tracing allows for the discrete visualization and quantification of axonal processes and their terminations. Although various types of non-fluorescent and fluorescent tracers are available, they are often limited by their use as a single bolus injection and, therefore, must be applied during an optimal temporal window for detection (reviewed by Kobbert et al., 2000 and Vercelli et al., 2000). The use of genetic vectors or transgenic mice to continually express reporter genes, such as green fluorescent protein (GFP), offers an alternative method to assess neuronal morphology and to anterogradely label axons over the course of weeks to months (for review, see Hechler et al., 2006, and Harvey et al., 2009a). In addition, reporter constructs can be combined with transgenes of interest, such as those that can improve the regenerative phenotype of the labeled neurons. In this way, the use of genetic vectors offers a powerful tool to assess axon regeneration as the somata of the anterogradely labeled axons also express the transgene of interest.

Adeno-associated viral (AAV) vectors are well characterized for inducing long-term transgene expression in numerous regions of the CNS with minimal immune response and toxicity (for review see Ruitenberq et al., 2002; Tenenbaum et al., 2004, Burger et al., 2005; Terzi and Zachariou, 2008). In the present study, recombinant serotype II AAV vectors were used due to their neuron specific tropism (Kaplitt et al., 1994; McCown et al., 1996; Mandel et al.,
AAV vector transgene expression is achieved through the intermolecular recombination of single-stranded genomes into episomal concatamers (Flotte et al., 1994; Duan et al., 1998). Given that each concatamer contains 3 to 6 genomes, co-infection of a cell with AAV vectors carrying different transgenes results in the co-expression of both genes (Yang et al., 1999). This characteristic of AAV may be exploited to co-localize the expression of GFP, as an anterograde tracer, together with transgenes of interest. The use of AAV vectors to express GFP has been demonstrated to fill individual neurons in a Golgi stain-like manner for at least twelve weeks (Chamberlin et al., 1998; Harvey et al., 2002). However, the AAV-mediated expression of GFP as an anterograde tracer for regenerating supraspinal axons has not been reported. Given that these axon populations project a relatively long distance from their somata, the goal of this study was to determine if GFP will distribute so that the distal ends of regenerating supraspinal axons may be easily visualized and quantified.

The complete transection of the thoracic spinal cord and implantation of a Schwann cell (SC) bridge is a rigorous model to assess axon regeneration in the CNS (reviewed by Bunge, 2008). This model was used in combination with the stereotaxic injection of AAV-GFP vectors into the brainstem to assess the ability of AAV-mediated GFP expression to anterogradely label regenerated supraspinal axons. AAV-GFP readily labeled axons that were observed in the rostral spinal cord and in the SC bridge. AAV-GFP-positive structures also were detected in the caudal spinal cord, distal to the bridge.
Methods

Experimental design

Twenty-four young adult (postnatal day 35) female Fischer rats, born on the same day, were obtained from Harlan Laboratories (Frederick, MD). They weighed approximately 160 grams at the time of injury. To ensure that transgene expression was maximal at the time of injury, rats were stereotaxically injected with AAV-GFP vectors between 2-6 weeks prior to the complete transection surgery and implantation of a SC bridge (Fig. 2.1a-d). In this way, the rats were injected with AAV vectors between the postnatal ages of 6 and 10 weeks, and at 12 weeks postnatally; all rats were transected/implanted with a SC bridge over the course of two days. Six weeks following the complete transection and implantation of a SC bridge, the rats were sacrificed for histological analysis (Fig. 2.1e). Two animals died during the stereotaxic injection surgery and two animals died during the transection/SC bridge implantation surgery. In addition, animals were excluded from the study based on the following blinded criteria: surgical damage to the spinal cord (n=2), a collapsed polymer channel that surrounded the cellular bridge (n=3), poor rostral stump insertion into the polymer channel (n=1), formation of a large cyst within the polymer channel (n=1), or poor GFP-labeling of brainstem axons (n=1). Finally, two animals were sacrificed for histological analyses two days after the complete transection/SC bridge implantation. In sum, ten animals were used for the quantification of GFP-labeled axons six weeks after the complete transection/SC bridge implantation.
Generation of AAV vectors

AAV vectors were generated by the Miami Project to Cure Paralysis Viral Vector Core, using the AAV Helper-Free System from Stratagene (La Jolla, CA). Briefly, 293 cells were grown to 70-80% confluency at which point they were transfected with the two helper plasmids and the transgene plasmid control for enhanced GFP (kindly provided by Dr. S. Whittemore, University of Louisville, KY) using jetPEITM (Poly-plus Transfection, San Marcos, CA). Transgene plasmids (Fig. 2.1f) were under the transcriptional control of the cytomegalovirus (CMV) promoter and contained a human growth hormone polyadenylation region (hGH-pA) and the Woodchuck post-transcriptional regulatory element (wPRE) to stabilize the mRNA and translation efficiency (Loeb et al., 1999; Xu et al., 2001). The cells and media were harvested 72 hours following the transfection and purified for virus using the AAV ViraKit from Virapur (San Diego, CA). The AAV titers were determined based on transducing units/mL (TU/mL), using HT1080 cells shocked with 0.8 µM camptothecin. Quantification of GFP-expressing HT1080 cells was performed to determine the titer of AAV-GFP (2.6 x 10^8 TU/mL).

Stereotaxic injection of AAV

The rats were first anesthetized by intraperitoneal injection of ketamine (45mg/kg) and xylazine (5 mg/kg). Next, the head was secured in a stereotaxic device attached to a micro-manipulator, and a sagittal incision was made to expose the cranial sutures. A micro-drill was used to create a heart shaped burr-hole caudal to lambda, exposing the dorsal aspect of the cerebellum as well as
the left and right transverse sinus (Fig. 2.1a). Bregma was used as the zero point for bilateral injections into the following regions: 2.5 mm lateral, 9.2 mm caudal, 11-10 mm as well as 7.6-7.1 mm ventral; 1.4 mm lateral, 9.6 mm caudal, and 10-6.8 mm ventral. 0.5 µL of vector was injected using a sp310i syringe pump (World Precision Instruments, Sarasota, FL) together with a 5 µL syringe modified with a 5 cm 33GA removable needle (Hamilton Company, Reno, NV) at a rate of 0.25 µL per minute for increments of 0.5 mm along the dorso-ventral axis. In this way, the following regions were injected bilaterally with: 2.5 µL to the ventro-lateral pons, 4.0 µL to the dorso-medial pons, and 3.0 µL to the ventro-medial rostral medulla, for a total of 19 µL in six injection sites. Injections were always performed in a specific order to minimize volume lesions (indicated in Fig. 2.1a). Following surgery, the rats received subcutaneous injections of 3 mL physiological saline twice a day (BID) for three days, to compensate for fluid loss, gentamicin (5 mg/kg) once a day (QD) for seven days to prevent infection, and the analgesic buprenorphine (0.1 mg/kg) three times a day (TID) for three days. Given that each stereotaxic injection surgery took approximately two hours, and in order to produce enough animals for statistically significant analysis, the stereotaxic surgeries were performed over the course of four weeks.

**Generation of purified SCs**

Purified (95-98%) SC populations were obtained from adult female Fischer rat sciatic nerves as described in part previously (Morrissey et al., 1991). Following dissection, nerve explants were placed in D10 [Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen Corporation, Carlsbad, CA; supplemented
with 10% fetal bovine serum and gentamicin (50 µg/mL)]. Contaminating fibroblasts were first removed by transferring the explants to new dishes weekly. Two weeks later, the nerve explants were incubated in 0.25 % dispase/0.05% collagenase in D10, dissociated by trituration, and plated. After reaching 90% confluency, the SCs were further purified by incubation in conditioned medium from mouse hybridoma cells containing the Thy1.1 antibody and adding rabbit complement to destroy fibroblasts. The SCs were expanded in D10 containing 20 µg/mL bovine pituitary extract (Invitrogen Corporation, Carlsbad, CA), 2.5 nM heregulin (Genentech, San Francisco, CA), and 2 µM forskolin. SCs were passaged to confluency three times at which point they were harvested by trypsinization and re-suspended in DMEM for transplantation.

**Spinal cord transection and SC bridge implantation**

The rats were anesthetized by intraperitoneal injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). A laminectomy was performed from thoracic vertebrae levels 7 (T7) to T9, with significant lateral exposure. Following dural removal from the exposed cord and cutting of the dorsal roots, a single incision was made with angled micro-scissors to completely transect the spinal cord at T8. Then the ventral dura was severed and the ventral roots were cut. This procedure led to the retraction of the spinal cord stumps approximately 2-3 mm (Fig. 2.1b). Completeness of the injury was confirmed by lifting the rostral and caudal stumps. A 5.0 mm long, polyacrylonitrile/polyvinyl chloride (PAN/PVC) polymer channel, with a 2.7 mm inner diameter, was positioned in the gap, accommodating rostral and caudal stump insertion approximately 1 mm into each
end of the channel. Then, $3.0 \times 10^6$ SCs in 15 µL DMEM were mixed with 10 µL of Matrigel (BD Biosciences, San Jose, CA) and immediately injected through the rostral of two holes previously created in the top of the channel (Fig. 2.1c). All SCs in this study were harvested from the same sciatic nerves and passaged at the same time. The SCs were kept on ice for no more than 2 hours before implantation. The muscle layers and skin were closed with sutures and Michel clips, respectively. Following the surgery, the rats received subcutaneous injections of 5 mL physiological saline BID for seven days, gentamicin (5 mg/kg) QD for seven days to prevent urinary tract infection, buprenorphine (0.1 mg/kg) TID for three days, and their bladders were expressed manually BID until they regained micturition.

**Tissue processing and immunohistochemistry**

The rats were sacrificed by terminal anesthesia (200 mg/kg ketamine and 20 mg/kg xylazine) and perfused transcardially with 20 USP units of heparin, then 200 mL 4°C, phosphate-buffered saline (PBS) followed by 400 mL, 4°C, 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains and spinal cords were extirpated and further fixed in PFA overnight and cryoprotected in PBS plus 30% sucrose and 0.025% sodium azide. Tissue blocks were embedded in PBS plus 12% gelatin and 0.025% sodium azide, and the blocks were quick frozen in crushed dry-ice. Brainstems were sectioned into six series at 40 µm parasagittally using a freezing microtome, and stained as free-floating sections before being mounted on slides. The SC bridges with rostral and caudal
spinal cords were serially sectioned into five series at 20 µm sagittally using a cryostat and mounted directly on slides.

The antigenic sites were blocked in PBS with 0.5% Triton-X plus 5.0% normal goat serum (NGS) and/or 5.0% normal donkey serum (NDS) and incubated overnight in the following primary antibodies: GFP (chicken, 3.19 mg/mL, 1:500, Chemicon, Temecula, CA), dopamine beta hydroxylase (DBH, mouse, 1:500, Chemicon), GFAP (SMI 22, mouse, 1:500, Covance, Denver, PA; rabbit, 1:500, DAKO, Carpinteria, CA), neurofilament (NF)-L (rabbit, 1:500, Encore, Alachua, FL), NF-M (rabbit, 1:500, Encore), NF-H (rabbit, 1:1000, Encore), and S100 (rabbit, 1:500, DAKO). Sections were rinsed in PBS and incubated in PBS plus 1% NGS and/or 1% NDS, plus 0.001% Hoechst and goat anti-mouse, goat anti-rabbit, and/or goat anti-chicken secondary antibodies conjugated to 488 or 594 fluorophores (1:200, Molecular Probes, Eugene, OR) and/or goat anti-mouse or goat anti-rabbit conjugated to Cy-5 (1:200, Jackson ImmunoResearch, West Grove, PA). Finally, the tissue was rinsed in PBS and cover-slipped with Vectashield mounting medium (Vector Labs, Burlingame, CA).

**Quantification of axon regeneration**

Adhering to guidelines established for the assessment of axon regeneration (Steward et al., 2003), sagittal sections of the SC bridge were analyzed via a line-transect method (Fig. 2.1e). The length of the polymer channel was determined first to serve as a reference for the distance between the rostral and caudal zero points, 0’ and 0”, respectively. Next, the extent of host spinal cord tissue inside the channel was defined by the expression of
GFAP-positive astrocyte somata, depicted in red at the spinal cord/SC bridge interface. The zero points, 0’ and 0”, were determined on the section that contained the tip of host spinal cord farthest from the rostral or caudal end of the polymer channel, respectively. This distance from the end of the polymer channel was then used to locate 0’ and 0” on the remaining sections. Then, dorso-ventral lines were drawn on each section along the rostro-caudal axis at -10.0 mm, 0.25 mm, 0.5 mm, 1.0 mm, 1.5 mm, 2.0 mm, and 2.5 mm from 0’ or 7.0 mm from 0”. To determine the dorso-ventral thickness of the bridge tissue, the total length of Hoechst staining was measured for each line inside the polymer channel. Then, the number of GFP-labeled axons (green lines) that crossed a given dorso-ventral line was determined, with the exception of the lines at 0’ and 0”. Thus, using the distance and thickness of the tissue measured, all axon counts were determined as either the number/mm² or the percentage of the labeled axons in the rostral spinal cord.

**Statistics**

All statistical analyses were performed with the Graph Pad Prism 5 software, using data from the experimental group of ten animals. These included one-tail nonparametric correlations and a one-tail Welch-corrected nonparametric t-test with 95% confidence intervals, as well as a two-way ANOVA.

**Results**

**AAV-GFP infects supraspinal brainstem neurons and labels their axons**

The stereotaxic injection of AAV-GFP resulted in the infection of numerous neuronal populations in the pons and medulla (Fig 2.2). In this way, AAV is
capable of creating a transgenic neuronal brainstem. In the brainstem, AAV-GFP only infected neurons (Fig. 2.3a-d) as GFP was not found in any cells that did not also express NeuN (compare 2.3b and d). AAV-GFP infected various neuronal populations, including the DBH-positive noradrenergic neurons in the brainstem (Fig. 2.3e-h). The intensity of GFP expression varied across brainstem neurons of similar morphology (Fig. 2.3a, d; arrowheads) and phenotype as depicted in the noradrenergic neurons of the locus coeruleus (Fig. 2.3e, h; arrowheads). GFP-labeled neuronal somata were not found outside the brainstem. Therefore, only targeted neuronal populations are infected and anterogradely traced with AAV-GFP.

Following injection of AAV-GFP into the brainstem, numerous GFP-labeled axon populations were detected throughout both the grey and white matter of the spinal cord rostral to the bridge from at least two (data not shown) to twelve weeks (Fig. 2.4a, b). Visualization of axons by fluorescence was further enhanced by combined use with anti-GFP immunohistochemistry. This allowed for the discrete and unambiguous visualization of axonal morphologies ranging from fine and beaded to robust. The number of GFP-labeled axons 10 mm rostral to the SC bridge varied across animals from 3200-6200 axons/mm². Neither the time interval following the stereotaxic injection of AAV (8-12 weeks; Fig. 2.4c) nor the postnatal age of the animal (6-10 weeks) at the time of injection (Fig. 2.4d) correlated with the number of GFP-labeled brainstem axons in a given animal’s spinal cord 10 mm rostral to the SC bridge. In sum, AAV-GFP is a
useful anterograde tracer for brainstem axon populations in the thoracic spinal cord.

**AAV-GFP anterogradely labels brainstem axons that regenerate into a SC bridge**

The injection of AAV-GFP into the brainstem followed by the complete transection of the thoracic spinal cord and implantation of a SC bridge, anterogradely labeled regenerating brainstem axons in the bridge (Fig. 2.5). By sectioning the SC bridge sagittally, and immunostaining for GFP, GFP-labeled axons were easily visualized crossing the bridge. GFP-labeled brainstem axons were often in close association with SCs (Fig. 2.6a, b). Similar to GFP-labeled somata, the intensity of GFP often varied in bridge axons that were of similar diameter and morphology (Fig. 2.6b). Axon regeneration was assessed at different distances across the SC bridge using a line-transect method of quantification in conjunction with computer-assisted fluorescent microscopy and Neurolucida (Figs. 2.1e and 2.5). The number of GFP-labeled axons that regenerated 2.5 mm into the SC bridge was not equivalent across animals and ranged from 0 to 123 axons/mm². Analogous to the variability in the number of GFP-labeled axons 10 mm rostral to the SC bridge, the length of time GFP was expressed did not correlate with the number of axons found to regenerate 2.5 mm into the SC bridge (Fig. 2.6c). However, as expected, the total number of GFP-labeled axons found to regenerate 2.5 mm into the SC bridge correlated with the total number of GFP-labeled axons present 10 mm rostral to the bridge (Fig. 2.6d, p<0.01). These data demonstrate that AAV-GFP may be used to
anterogradely trace the regeneration of brainstem axons in the thoracic spinal cord.

In an attempt to normalize for the differences in the number of GFP-labeled axons across animals, the quantification of GFP-labeled axons on the bridge also was determined as a percent of the GFP-labeled axons present 10 mm rostral to the spinal cord/SC bridge interface. The percentage of GFP-labeled axons that regenerated proximally (0.25 mm and 0.5 mm) into the SC bridge was similar across animals with different numbers of GFP-labeled axons in the rostral spinal cord (Fig. 2.6e). However, animals with a total number of 3,200-4,900 GFP-labeled axons/mm² in the rostral cord exhibited a significantly lower percentage of those axons found to regenerate farther, 1.0 mm, 1.5 mm, 2.0 mm, and 2.5 mm, across the bridge compared to animals with a total number of 4,900-6,200 GFP-labeled axons/mm² in the rostral cord (Fig. 2.6e; p<0.05, two-way ANOVA). Animals with a total number of 3,200-4,900 GFP-labeled axons/mm² in the rostral cord exhibited an average of 0.3% of their axons to regenerate 2.5 mm across the bridge compared to an average of 1.6% in animals with a total of 4,900-6,200 GFP-labeled axons/mm² in the rostral cord (Fig. 2.6e; p<0.01, Welch-corrected nonparametric t-test). Thus, the percentage of GFP-labeled axons found to regenerate distally into a SC bridge was not equivalent in all animals and was dependent upon the number of GFP-labeled axons in the rostral spinal cord.
AAV-GFP labeled axon-like structures are present in the caudal spinal cord distal to the SC bridge

Injection of AAV-GFP into the brainstem resulted in the presence of GFP-labeled axon-like structures in the caudal spinal cord at six weeks following the complete transection of the thoracic spinal cord and implantation of a SC bridge (Fig. 2.7a). These structures are not likely to be regenerating brainstem axons because they could not be retrogradely traced (data not shown), and they were present two days after injury (Fig. 2.7b). They were likely labeled in the 14-42 day period before spinal cord transection. Increasing time of GFP expression during this period strongly correlated with the total number of GFP-labeled structures in the caudal cord (p<0.001; Fig. 2.7c). This correlation was distinct from that of the GFP-labeled axons in the rostral spinal cord or those that regenerated into the SC bridge, neither of which correlated with the duration of GFP expression. The nature of these GFP-labeled structures will be the focus of a subsequent report.

Discussion

The use of AAV-GFP as an anterograde tracer

Following the infection of neurons with AAV-GFP, long-term stable expression allows GFP to distribute through the entire neuron, thereby enabling the discrete and unambiguous visualization of distal axons of varying morphologies. The visualization of axons by fluorescence AAV-GFP was further enhanced by the combined use with anti-GFP immunohistochemistry, and allowed for the detection of numerous additional axons that may have been labeled but not have otherwise been detectable. The half-life of wild-type GFP in
many mammalian cells has been reported to be approximately 26 hours (Corish and Tyler-Smith, 1999). However, in the current study the presence of enhanced GFP at the distal ends of regenerating brainstem axons in the bridge and for up to six weeks in axon-like structures distal to the site of complete transection, which suggests that the half-life of the enhanced protein may be much longer in axoplasm or another mechanism may be involved. Klein et al. (2002) reported a dose-dependent effect of AAV on the expression of GFP, and thus variations in the titer of AAV-GFP are likely to have direct effects on the number of axons that may be anterogradely traced from a discrete region of the CNS.

Variations in the number of GFP-labeled axons in the rostral spinal cord reported here are likely due to slight, uncontrollable differences in the stereotaxic injection surgery, such as resultant edema and local hemorrhage that may affect AAV-GFP distribution in the parenchyma, and the precise level of the head in the stereotaxic device. Although the titer of injected AAV-GFP was equivalent in all animals, these variations dictate the amount of AAV that is delivered to a given population of brainstem neurons, and thus the relative expression level of GFP in their somata and axons. Despite these potential differences, the lack of an observed correlation between the postnatal age of the animal at the time of stereotaxic injection and the number of GFP-labeled axons suggests that the stereotaxic coordinates for brainstem neurons of postnatal rats from six to ten weeks of age do not change significantly.
The use of AAV-GFP to assess axon regeneration

AAV-GFP offers a new method to anterogradely trace and quantify the regeneration of axons. The continual expression of GFP is distinct from the bolus injection of more classical tracers, and revealed several new variables concerning the assessment of axon regeneration with a fluorescent tracer. These variables include the length of time GFP is expressed, differences in the accumulation of GFP along an axon (dependent upon the relative expression levels in the somata), and the presence of long-lasting GFP-positive structures distal to the site of injury.

The length of time GFP was expressed correlated with the number of GFP-labeled structures/axons in the spinal cord caudal but not rostral to the SC bridge. The discrepancy between these two regions of the spinal cord may be partially explained by the fact that unlike the rostral spinal cord, the caudal structures were no longer connected with the somata after the complete transection. In this way, GFP was only capable of distributing to the caudal spinal cord for two to six weeks, but in the rostral spinal cord GFP could be distributed to the axons for eight to twelve weeks. This suggests that a correlation between length of time GFP was expressed and the number of GFP-labeled axons may only be apparent during the first six weeks. If this were true, and the complete transection was performed eight to twelve weeks after the injection of AAV-GFP into the brainstem, there may not be a correlation between the time of GFP expression and the number of GFP-labeled structures in the caudal spinal cord.
An alternative explanation for the observed difference between the rostral and caudal spinal cord, may be due to the region of the axon being labeled, whereby the distal ends of axons are more affected by the duration of GFP expression. According to this suggestion, there should be a correlation with the length of time GFP was expressed and the number of GFP-labeled axons found to regenerate into the distal end of the SC bridge, i.e. 2.5 mm. However, no such correlation was observed, and this is likely due to the fact that all of the animals received a complete spinal cord transection on the same day. Therefore GFP was able to distribute into the regenerating portion of the axons for the exact same amount of time, i.e. six weeks, unlike the caudal spinal cord.

If the observed differences between the rostral and caudal spinal cord discussed above are dependent upon the portion of the axon being assessed, this suggests that GFP may not be uniformly distributed throughout the axoplasm. An example of such a process comes from proteins that sense neurite length. These proteins are not distributed evenly throughout the axoplasm due to rapid anterograde vesicular transport and slow retrograde diffusion (Toriyama et al., 2010), and GFP may accumulate at the distal ends of supraspinal axons in an analogous fashion. This possibility of non-uniform distribution is supported by the observation that depending upon the number of GFP-labeled axons rostral to the bridge, the percentage of those axons that regenerate into a SC bridge is similar proximally (0.25 mm and 0.5 mm), but differs distally (1.0 mm, 1.5 mm, 2.0 mm, 2.5 mm). In this way, animals with a greater number of GFP-labeled axons in the rostral spinal cord, likely due to
variable infection of brainstem neurons during the stereotaxic injections, exhibit a higher percentage of those axons found in the distal end of the bridge. Therefore, neurons that express high levels of GFP may have more GFP accumulation at the distal end of their axon.

If GFP does distribute non-uniformly along an axon, then careful experimental design would be required when using AAV as both an anterograde tracer and a method to test the effects of transgene expression on axon regeneration. First, only animals with comparable times of GFP expression should be compared across groups. More importantly, the titers of the injected AAV vectors must be equivalent across groups. Titering of AAV by transduction units/mL is the most accurate way to determine the amount of functional AAV particles (Xiao et al., 1997). However, this method often relies on the visualization and quantification of transgene expression, and therefore equivalent transduction titers of AAV vectors with different transgenes may not have the same number of functional particles. Since the genome size of AAV often limits the use of an internal ribosome entry site or bi-cistronic promoters, an alternative method such as the 2A polyprotein strategy could be employed (Furler et al., 2001; Szymczak and Vignali, 2005). For this strategy, a short 18 amino acid long sequence, identified in the picornavirus, is inserted between transgenes. The 2A sequence causes the ribosome to pause during peptide synthesis and thereby produce multiple proteins from a single mRNA (Donnelley et al., 2001). This allows for two transgenes of interest to be produced with consistent equimolar translation efficiency (Szymczak et al., 2004; Ibrahimi et al., 2009), with
comparable transduction units/mL across different AAV vectors (Tang et al., 2009).

An intriguing observation made using AAV-GFP to anterogradely trace brainstem axons for up to six weeks prior to spinal cord injury was the presence of GFP-positive structures in the caudal spinal cord at least six weeks after injury. The presence of axon-like structures may confound the assessment of axon regeneration because they may be mistaken for regenerating axons. It is unlikely that they are regenerating because they were visible two days after transection and could not be retrogradely traced. Awareness of these remnant structures is important in studies that use AAV as an anterograde tracer as well as a method to express a transgene of interest prior to the time of injury.

The use of AAV as an intrinsic treatment strategy to express transgenes that regulate axon regeneration

Following injury to the CNS, functional recovery and repair will ultimately require a combination of treatment strategies, including the modification of intrinsic mechanisms to enhance the regeneration of axons. Recently, many of the genes that regulate axon regeneration have been elucidated (Bulsara et al., 2002; Park et al., 2008; Moore et al., 2009; Blackmore et al., 2010), and their expression can be regulated by the use of genetic vectors. Towards this end, AAV vectors have been used by several groups to deliver transgenes to the somata of injured neurons in the CNS, with the goal of improving the regeneration of their axons through the expression of genes such as growth factors and BCL-2 (Pernet et al., 2005; Leaver et al., 2006a, b; Planchamp et al., 2008; Harvey et al., 2009b). A limitation for many of these studies is the use of
classical tracers or immunohistochemical markers such as GAP-43 for the quantification of axon regeneration as opposed to the expression of a reporter gene such as GFP. Thus, the axon regeneration observed with traditional tracers or markers may not necessarily result from neurons expressing the transgene of interest. In order to surmount this issue, the use of genetic vectors such as AAV, will be required to identify and quantify regenerating axons expressing a transgene of interest.

The use of AAV is a powerful tool when different vectors are combined; the transgene from one vector may function as an anterograde tracer and the other vector may express a transgenes of interest. However, studies which use AAV-GFP to assess axon regeneration must be mindful of the potential for GFP to be non-uniformly distributed along the axon. In addition, when experimental designs require infection with AAV-GFP prior to injury, GFP may be retained in structures distally and these structures must be distinguished from regenerating axons. Nevertheless, the use of AAV-GFP allows for easy visualization and quantification of axon regeneration. Furthermore, true differences in the amount of regeneration across experimental groups may be directly attributed to the neuronal expression of a given transgene. Thus, AAV is a promising new method for the study of intrinsic mechanisms that regulate axon regeneration.
Figures and legends

Figure 2.1: Experimental design. a) Photograph of the burr-hole created for the stereotaxic injections of AAV-GFP, illustrating the sites and numerical order of the injections (numbers; scale bar=1 mm). b) Photograph of the complete spinal cord transection (scale bar=1 mm). c) Photograph of the SC bridge implantation, showing the dorsal aspect of the rostral and caudal spinal cord stumps inserted into a polymer channel. Two holes (arrows) in the channel were used for the injection of an initially fluid mixture of SCs and Matrigel (scale bar=1 mm). d) The experiment timeline. Rats were stereotaxically injected between 42 and 14 days prior to the complete transection and implantation of a SC bridge, at time point zero. Animals were maintained for an additional 42 days. e) The line-transect method was employed for quantification of GFP-labeled axons (green) in a SC bridge. The polymer channel (thick black lines) and the transverse lines used for quantification (thin purple lines) are diagrammed. The numbers represent mm from the rostral interface. In this illustration and all subsequent figures, rostral cord is left, caudal cord is right, dorsal cord is top, and ventral cord is bottom. f) Expression cassettes for the AAV vectors, flanked by a left and right internal terminal repeat (ITR). The cassette contained a cytomegalovirus (CMV) promoter, a human beta-globin intron (β-Globin Intron), an enhanced GFP (eGFP) sequence, a woodchuck posttranslational element (wPRE), and a human growth hormone poly-adenylation (hGH-pA) region.
Figure 2.2: AAV-GFP induces transgene expression in numerous brainstem neuron populations. Following the stereotaxic injection of AAV-GFP, various brainstem neurons express GFP (green), including DBH-positive neurons (red). Hoechst stained nuclei are blue (scale bar=1 mm). The dashed boxed in the medulla delineates the higher magnification inset picture, where DBH-positive neurons are indicated with arrowheads (scale bar=20 µm).
**Figure 2.3: In the brainstem, AAV-GFP only infects neurons.** GFP (a) is found only in NeuN positive neurons (b). Hoechst stained nuclei (c) and the merged confocal image (d) shows cytoplasm and nuclei indicative of co-localization. Note that neighboring neurons of similar morphology exhibit different intensities of GFP labeling (arrowheads). AAV-GFP also infects noradrenergic brainstem neurons showing GFP expression (e), DBH expression (f), Hoechst stained nuclei (g) and a merged confocal image (h). Following the injection of AAV-GFP, noradrenergic neurons also exhibit different intensities of GFP labeling (arrowheads; scale bar=5 µm).
Figure 2.4: The stereotaxic injection of AAV-GFP into the brainstem functions to anterogradely trace brainstem axons in the thoracic spinal cord. Hundreds of GFP-labeled axons (green) are present 10 mm rostral to the SC bridge in both lateral (a) and medial (b) spinal cord (scale bar=500 µm). GFAP immunostaining is red and the central canal is indicated by an arrowhead. In animals injected with AAV-GFP, there is no correlation between the number of GFP-labeled axons in the rostral spinal cord and the length of time GFP was expressed (c) or age of the animal (d).
Figure 2.5: The stereotaxic injection of AAV-GFP into the brainstem functions to anterogradely trace brainstem axons that regenerate into a SC bridge. A confocal image of a SC bridge, showing GFP-labeled axons (green, arrows) together with immunostaining for GFAP (red) to delineate the rostral spinal cord stump. The polymer tube is indicated by arrowheads and transverse lines (white) illustrate those used in the line-transect method for axon quantification. SCs immunostained for S100 (blue) are seen throughout the bridge as well as through a hole in the polymer channel at the top right. Red clusters of autofluorescent macrophages are indicated by asterisks (scale bar=0.5 mm).
**Figure 2.6: GFP may not be uniformly distributed along a regenerating axon.** GFP-labeled axons (green, arrows) 1.0 mm (a) and 2.5 mm (b) beyond the rostral interface are in close association with S100-positive SCs (blue; scale bar=20 µm) are shown. Note the difference in the intensity of GFP expression between axons of similar diameter and morphology in b (red arrows). The inset indicates where the higher magnification images were obtained (scale bar=1 mm). c) The number of GFP-labeled axons that regenerated to the distal end of the SC bridge (2.5 mm) did not correlate with the length of time GFP was expressed. d) The number of GFP-labeled axons that regenerated to the distal end of the SC bridge (2.5 mm) correlated with the number of GFP-labeled axons 10 mm rostral to the bridge (p<0.01; r²=0.4). e) The percentage of GFP-labeled axons that regenerated to more proximal distances (0.25 mm and 0.5 mm) was similar among animals with a range in GFP-labeled axon number (3,200-6,200 axons/mm²). However, the percentage of axons found to regenerate to the distal end of the SC bridge (1.0, mm 1.5 mm, 2.0 mm, and 2.5 mm) was less in animals with fewer GFP-labeled axons 10 mm rostral to the bridge (3,200-4,900 axons/mm²) than in animals with more GFP-labeled axons (4,900-6,200 axons/mm²; *=p<0.05; two-way ANOVA). The percentage of axons found to regenerate to the distal end of the bridge (2.5 mm) was five-fold less in animals with fewer GFP-labeled axons 10 mm rostral to the bridge than in animals with more GFP-labeled axons (*=p<0.05; Welch-corrected nonparametric t-test; error bars=SEM).
**Figure 2.7:** The stereotaxic injection of AAV-GFP into the brainstem followed by the complete transection of the thoracic spinal cord and implantation of a SC bridge labels structures in the caudal spinal cord. GFAP-positive astrocytes (red) and GFP-labeled structures (green, arrows) in the caudal spinal cord six weeks (a) or two days (b) after the complete transection. Note the dashed nature of the GFP-labeled structures (scale bar=20 µm). c) The number of GFP-labeled structures found 7 mm caudal to a SC bridge correlated with the time GFP was expressed between stereotaxic injection and complete transection/SC bridge surgeries (p<0.001; $r^2=0.5$).
C

$\#$ GFP-labeled structures/mm$^2$

0 10 20 30 40 50
Days between injection of AAV-GFP & complete transection/SC bridge implantation
CHAPTER 3

EXPRESSION OF THE DEVELOPMENTAL TRANSCRIPTION FACTOR, MASH-1, IN THE BRAINSTEM ENHANCES NORADRENERGIC AXON REGENERATION AND IMPROVES HINDLIMB MOVEMENTS AFTER COMPLETE SPINAL CORD TRANSECTION/IMPLANTATION OF A SCHWANN CELL BRIDGE
Overview, background, and results

Unlike mature CNS neurons, embryonic and perinatal neurons can regenerate their axons after injury. We report that expression of a developmentally regulated transcription factor, mammalian achaete-scute homolog-1 (MASH-1), in mature brainstem neurons enhances noradrenergic axon regeneration and improves hindlimb joint movements, following complete spinal cord transection and implantation of a Schwann cell (SC) bridge. Therefore, when adult neurons expressed a transcription factor that is transiently expressed during their development, they became more capable of axonal regeneration.

Axonal regeneration after spinal cord injury (SCI) will likely require a combination of treatments. Implanted SCs provide a supportive environment for axonal regeneration which is enhanced by additional extrinsic treatments such as neurotrophins or chondroitinase (Bunge, 2008). However, in order to achieve the most robust axon regeneration after SCI, the intrinsic state of adult neurons also may need to be changed. This may be accomplished through conditioning lesion paradigms (Richardson and Issa, 1984), the application of neurotrophins to the somata (Kobayashi et al., 1997), elevation of intracellular cAMP (Pearse et al., 2004), and manipulations of the PTEN/mTOR pathway (Liu et al., 2010). Furthermore, axonal elongation is transcription-dependent (Smith and Skene, 1997), and neuronal maturation to a non-regenerative phenotype is in part regulated by transcription factors such as the KLF family (Moore et al., 2009). Thus, the manipulation of factors regulating gene transcription during
axonogenesis may shift a neuron’s phenotype to a more regeneration-capable state after SCI.

Many transcription factors that regulate the development of neurons in the CNS are well characterized, including those of the basic helix-loop-helix (bHLH) family. Of these, the class II bHLH transcription factor, MASH-1, transiently expressed in subsets of neural progenitor cells as they exit the mitotic cycle, is essential for the determination and differentiation of noradrenergic brainstem neurons (Johnson et al., 1990). Furthermore, MASH-1 regulates the expression of genes associated with axon regeneration (Lo et al., 1998; Hamada et al., 2006) and is capable of trans-differentiating adult fibroblasts into neuron-like cells (Vierbuchen et al., 2010).

To determine if the expression of MASH-1 in adult noradrenergic brainstem neurons would enhance the regeneration of their axons after SCI, the brainstems of rats were injected with either AAV-GFP (control) or AAV-GFP plus AAV-MASH-1 (treated) between six and two weeks before the complete transection/implantation of a SC bridge. The animals survived for an additional six weeks, and were subjected to an open-field hindlimb locomotion test, the BBB (Basso et al., 1995, 1996). After six weeks, the animals were euthanized and noradrenergic axons in the SC bridge were immunostained and quantified. The experimental design was described previously (see Chapter 2, page 82).

In treated animals, MASH-1 was expressed in numerous populations of brainstem neurons, including those of the noradrenergic phenotype, as evident by MASH-1 protein co-localized with dopamine-beta hydroxylase (DBH; Fig.
3.1a-d). Treated animals exhibited more axon regeneration into and across the SC bridge compared to controls (Fig. 3.2a-h); an average of 97 +/-30.5 DBH axons/mm² regenerated 0.25 mm into the bridge compared to 38 +/-10.5 DBH axons/mm² in controls and an average of 12 +/-5 DBH axons/mm² regenerated 2.5 mm across the bridge compared to 5 +/-3 DBH axons/mm² in controls. In treated animals many of the regenerating DBH-positive axons also expressed GFP (Supplementary Fig. 3.1) indicating the expression of MASH-1 in their somata. Whereas no difference was found in DBH-positive axon number rostral to the bridge, the percentage of those axons that regenerated was significantly increased in treated animals compared to controls (Fig. 3.2h).

During the first six weeks, treated animals compared to controls exhibited improved scores on a modified BBB scale (Ferguson et al., 2004; Fig. 3.2i). At week six, 90% of the treated animals exhibited extensive movement of all three joints in both hindlimbs (Fig. 3.2j). Not all animals were subjected to histological analysis due to various exclusion criteria such as poor gross morphology of the bridges (see Chapter 2, Methods). Nevertheless, analysis of all 32 animals subjected to the BBB test revealed that treated animals exhibited improved scores over the first six weeks (Supplementary Fig. 3.2).

This study demonstrates that expression of a developmentally regulated transcription factor in adult CNS neurons creates a more immature-like phenotype capable of axon regeneration and recovery of function. More robust effects might be achieved through manipulations of negative regulators of the bHLH family implicated in axonal growth (Vinals et al., 2004; Lasorella et al.,
2006), analogous to the KLF family (Moore et al. 2009), as well as combination with other transcription factors to form synergistic complexes, or modification of epigenetic mechanisms that “open” silenced promoter and/or enhancer regions.

**Methods**

**Experimental design and generation of AAV**

Details of these methods are presented in Chapter 2, pages 82-83, with the addition that AAV-MASH-1 vectors were generated using a transgene for MASH-1 (kindly provided by Dr. Anderson, California Institute of Technology). The MASH-1 transgene cassette was sub-cloned by first excising the GFP transgene and then inserting the MASH-1 transgene in the same location. Equivalent volumes of AAV were injected in both control and treated animals. The control animals received AAV-GFP, with a HT1080 cell expression titer of $2.6 \times 10^8$ TU/mL, and the treatment animals received a 1:1 mixture of AAV-GFP and AAV-MASH-1, with HT1080 cell expression titers of $2.6 \times 10^8$ TU/mL and $1.0 \times 10^8$ TU/mL, respectfully.

**Stereotaxic injection of AAV, generation of purified SCs, and spinal cord transection and SC bridge implantation**

Details of these methods are presented in Chapter 2, pages 83-86.

**Tissue processing and immunohistochemistry**

Details of these methods are presented in Chapter 2, pages 86-87, with the addition of the additional primary antibody, MASH-1 (rabbit, 1:200, Santa Cruz, CA).
Quantification of noradrenergic axon regeneration

Details of these methods are presented in Chapter 2, pages 87-88, with the exception that DBH positive, not GFP-labeled axons were quantified.

Assessment of hindlimb locomotor function

To assess improvement in hindlimb motor function, animals were subjected to a four minute open-field locomotor test, the BBB. This popular test for assessing motor function after spinal cord injury is described in detail by Basso et al. (1995, 1996). All rats were tested one week prior to the stereotaxic injection surgery as well as one week prior to the complete transection and implantation of a SC bridge. Any rats not receiving the maximum score at these pre-injury time points were removed from further study. The rats were then tested on a weekly basis, for six weeks after the complete transection and implantation of a SC bridge. In the final week, the rats were video-recorded while being tested. To enhance the metric properties of the scale and to assure the scores were continuous and ordinal, the scores were converted from the 21 point scale to a modified 12 point scale (Ferguson et al., 2004).

Statistics

All statistical analyses were performed with the Graph Pad Prism 5 software, using data from twenty-one animals that were subjected to DBH-positive axon quantification and scored on the hindlimb locomotion test. In addition, an analysis was performed on all 32 animals that were scored on this test. These analyses included two-way ANOVAs with Bonferroni post hoc tests, a Chi-squared test, and a Cohen’s test for effect size.
Discussion

MASH-1 expression in noradrenergic brainstem neurons

This novel study induced adult brainstem neurons to express a transcription factor that is transiently expressed during their development. Following the stereotaxic injection of AAV-GFP plus AAV-MASH-1, MASH-1 expression was observed in numerous brainstem populations, including noradrenergic neurons. MASH-1 expression as assessed by immunohistochemistry was not easily discernable, however, in a majority of the noradrenergic neurons that expressed GFP. Noradrenergic neurons did not express robust levels of GFP compared to other brainstem populations and this may be due to a diminished infectivity of noradrenergic neurons by AAV. Thus, the relatively low levels of transgene expression by most noradrenergic neurons may have resulted in low levels of MASH-1 protein that are not detectable by immunohistochemistry. However, the example of MASH-1 protein expression in a noradrenergic neuron depicted in Fig. 3.1 was obtained from a noradrenergic neuron with relatively robust expression of GFP. Because the expression of the MASH-1 transgene in this noradrenergic neuron also was likely to be more robust than in other noradrenergic neurons, the protein may have been easier to detect by immunohistochemistry.

Two types of controls were generated for the MASH-1 immunostaining. The first used tissue sections from treated animals that were only immunostained with goat anti-rabbit secondary antibodies conjugated to a 594 fluorophore. The second used both control and treated tissue that was not immunostained for
either primary or secondary antibodies. Careful analysis of these immunostaining controls, on two separate microscopes, revealed that some of the more robust GFP-positive neurons emitted a weak signal upon excitation using the 594 filter on either microscope. This bleed-through effect is likely caused by the relatively broad excitation spectrum of GFP. Despite the potential for such bleed-through in treated animals immunostained for MASH-1, the MASH-1 protein signal was discernable when a more intense 594 filter signal was present in regions of the neuron, such as the nucleus, where the GFP signal from the 488 filter was not as robust. For example, in Fig. 3.1, the red signal is not present through the entire GFP-positive region of the cytoplasm and axon, suggesting that the more intense red signal is not bleed-through and is at least in part derived from MASH-1-labeling. Thus, the MASH-1 protein is likely expressed in some of the noradrenergic neurons infected with AAV, but robust GFP expression may produce a faint 594 filter signal.

An alternative explanation for limited detection of MASH-1 in noradrenergic neurons may be due to the expression of negative bHLH regulators in noradrenergic neurons. Several negative bHLH regulators, such as HES and Id subfamily members, are up-regulated after SCI (Kabos et al., 2002). These proteins sequester the ubiquitous bHLH binding partner E47, which leads to the degradation of MASH-1 and other class II bHLH proteins via ubiquitination (Vinals et al., 2004; Lasorella et al., 2006; Wang et al., 2006; Mehmood et al., 2009). Interestingly, the Id subfamily members also have been found to regulate axonal growth (Konishi et al., 2004), and this likely occurs through sequestering
other bHLH proteins. In order to determine if these factors are limiting the expression of MASH-1 protein in noradrenergic neurons, they must first be found to co-localize with MASH-1 and DBH. Then, if these repressors were indeed regulating MASH-1 protein function and stability, co-infection of noradrenergic neurons with AAV-MASH-1 and AAV-Phox2b may reduce their expression, given that Phox2b represses Id2 and HES5 expression during development (Dubreuil et al., 2002). A more direct approach to reduce the expression of Id and HES proteins also may be accomplished through the use of siRNA, and in this way possibly increase the number of DBH-positive neurons that express robust levels of MASH-1 protein.

In addition to the Id and HES proteins, microRNA (miR) may limit the expression of MASH-1 protein. For example, miR-23b regulates the class VI bHLH HES-1 (Kimura et al., 2004) and miR-1 regulates the class II bHLH MyoD (Zhao et al., 2005). Although no specific miR for MASH-1 have been described in the literature yet, candidate miRs for MASH-1 may be predicted through the use of computational methodologies of the MASH-1 transgene or identified through deep sequencing of the transcriptome (reviewed by Majer and Booth, 2010) of cells which transiently express MASH-1 during their differentiation, i.e. PC12 cells. Once these candidates are identified they must be demonstrated to reduce MASH-1 transgene and protein levels as well as reduce expression of a MASH-1-regulated reporter such as luciferase. Then, in vivo, the expression of their antisense may result in enhanced expression of MASH-1 protein in noradrenergic neurons. With respect to the current results, the forced expression
of MASH-1 by AAV may produce enough protein to have a molecular effect, but negative regulators may diminish the protein levels to below the threshold of detection in most noradrenergic neurons. Nevertheless, given the specific effect of MASH-1 expression on noradrenergic neurons, i.e. the enhanced regeneration of their axons, it is highly likely that some amount of MASH-1 protein was expressed noradrenergic neurons infected with AAV.

The expression of MASH-1 in the brainstem enhances the regeneration of noradrenergic axons into and across a SC bridge

The expression of MASH-1 in the brainstem significantly increased the regeneration of noradrenergic axons at all measured distances into a SC bridge (Fig 3.2g, h). Also, the Bonferroni post hoc test revealed a significant increase in axon regeneration at 0.25 mm. This suggests that MASH-1 may affect axon regeneration through the potentially inhibitory environment of the rostral host spinal cord/SC bridge interface. Given that MASH-1 expression reduces the expression of Nogo receptor (Hamada et al., 2006), the expression of MASH-1 may be limiting the effects of factors known to inhibit axon regeneration, such as chondroitin sulphate proteoglycans (CSPGs) and proteins in myelin. Alternatively, MASH-1 may enhance survival and reduce dieback of the noradrenergic axons at the rostral interface. Thus, more noradrenergic axons may be present at a given interface for potential regeneration into the bridge. Although no difference was observed between the number of noradrenergic axons 10 mm rostral to the interface, the possibility remains for a difference closer to the interface.
The observed increase in axon regeneration into and across the SC bridge is likely due to a direct effect of MASH-1 expression in noradrenergic neurons; although an indirect effect on the noradrenergic neurons cannot be ruled out. For example, it is possible that MASH-1 expression may induce neighboring brainstem neurons to produce paracrine factors that act on noradrenergic neurons to enhance the regeneration of their axons. Given that such a mechanism has yet to be described, it is more likely that MASH-1 is having a more direct effect on axon regeneration.

An observation that supports a direct effect for MASH-1 is the co-localized expression of GFP with DBH-positive axons that regenerated into the SC bridge in treated animals but not in control animals. However, non-GFP-labeled noradrenergic axons were observed to regenerate in both control and treated animals. Together, this suggests that the additional regeneration of noradrenergic axons in treated animals is due to their infection with AAV-GFP plus AAV-MASH-1 in their somata, which is the strength of using AAV-GFP as an anterograde tracer as described in Chapter 2. The absence of GFP expression in some of the DBH-positive axons that regenerate into the SC bridge, however, may be due to a relatively low level of AAV mediated transgene expression in noradrenergic neurons compared to other brainstem populations. Given this relatively low level of AAV mediated transgene expression in noradrenergic neurons, endogenous GFP expression is not easily detectable in their axons and requires anti-GFP immunohistochemistry. Nevertheless, even with anti-GFP immunohistochemistry some noradrenergic neurons infected with AAV-GFP may
still have axons below the threshold for detection. Another reason GFP was not detected in some DBH-positive axons that regenerated into the SC bridge may be that their somata were not infected with AAV. Despite the presence of DBH-positive axons that were not labeled with GFP, treated animals did exhibit GFP co-localization with some of the DBH-positive axons that regenerated into the SC bridge and control animals did not. Thus, MASH-1 transgene expression is likely having a direct effect on the regeneration of some noradrenergic axons.

MASH-1 may directly affect the intrinsic state of noradrenergic neurons through binding to E-box motifs in cis-regulatory regions of regeneration-associated genes (RAGs). In this way, MASH-1 expression may regulate gene expression patterns analogous to those that occur during development. Will recapitulating development be required for axon regeneration? In mammals, conditioning lesions trigger a recapitulation of some developmental patterns of gene expression, such as growth-associated protein-43 (GAP-43) and tubulin 1a (Tub1a; Hoffman and Cleveland, 1988; Hoffman, 1989). In contrast, some RAGs are not expressed in mammalian CNS neurons during development but only during axon regeneration (Bonilla et al., 2002; Tanabe et al., 2003). Furthermore, different signaling pathways may mediate the developmental-versus conditioning lesion-induced axon growth in mammals (Liu and Snider, 2001). Thus, when axons of mature neurons do exhibit a capacity for regeneration in the mammalian CNS, they sometimes recruit new genes and mechanisms as opposed to recapitulating those from development. This suggests that recruiting new mechanisms to induce axon regrowth may be less
difficult for many mature CNS neurons to accomplish than surmounting the factors that prevent them from recapitulating developmental gene expression patterns.

Differences in the expression of RAGs during development and axon regeneration are mediated by cis-regulatory regions in a species specific manner. In the zebrafish, proximal cis-regulatory regions regulate the developmental expression of GAP-43 and Tub1a, but these regions are not necessary for re-expression of these RAGs in adult CNS neurons that are intrinsically capable of axonal regeneration (Udvadia et al., 2001, 2008; Goldman and Ding, 2000; Goldman et al., 2001; Senut et al., 2004). Although proximal cis-regulatory regions that mediate developmental expression of many RAGs are conserved between teleosts and mammals, the distal regions for GAP-43 and Tub1a are only conserved in teleosts that are capable of regenerating their CNS axons and are not conserved in mammals (Kusik et al., 2010). Interestingly, in teleosts, these distal cis-regulatory regions contain E-box motifs as well as homeobox motifs (Fujimori et al., 2008; Kusik et al., 2010), but the particular transcription factors that bind to those motifs during axon regeneration remain to be determined. Nevertheless, deficient expression of RAGs after injury to adult mammalian CNS neurons may be due to either the absence of necessary transcription factors for proximal cis-regulatory regions or the absence of necessary motifs conserved in the distal cis-regulatory regions of teleosts. Given that MASH-1 is not expressed after injury to the mammalian CNS, the observed increase in axon regeneration in the current study suggests that an absence of
transcription factors in mammals may be partially responsible for the deficient expression of RAGs. Therefore, MASH-1 expression in the current study may be binding to the proximal cis-regulatory regions of RAGs and thereby recapitulating developmental patterns of gene expression that induce axon regeneration.

G/C-rich binding sites for Krüppel-like factors (KLFs) are also present in the distal cis-regulatory region of Tub1a in teleosts, but are not conserved at the same site in mammals, although isolated G/C-rich sites do exist in the distal regions (Kusik et al., 2010). These isolated sites in mammals may be responsible for the KLF transcription factor-mediated retinal ganglion cell (RGC) axon regeneration reported by Veldman et al. (2007) and Moore et al. (2009). Furthermore, KLF transcription factor binding to G/C-rich cis-regulatory regions is required for both development and regeneration of RGC axons (Veldman et al., 2007; 2010). Together, the results from the studies just discussed and the current study suggest that the recapitulation of development may not be necessary, but may be sufficient to enhance axon regeneration.

This study demonstrates that axon regeneration may be enhanced by factors, such as MASH-1, that also mediate states of differentiation. Expression of bHLH proteins are fundamental for somatic cells to de-differentiate into a more immature-like state in their lineage and/or trans-differentiate into a different lineage (reviewed by Selvaraj et al., 2010). The expression of the class II bHLH transcription factor, MATH-1, is capable of trans-differentiating both postnatal and adult non-sensory cells into cochlear hair-like cells in vivo (Zheng and Gao,
In addition, the expression of the class II bHLH, NeuroD, trans-differentiates cells in diabetic mice livers into islet-like cells that increase expression of pro-insulin processing enzymes and other islet specific transcripts in vivo (Kojima et al., 2003). More recently, the de-differentiation of primary cultures of adult human fibroblasts into pluripotent stem cells was, in part, mediated by the class III bHLH protein, c-Myc (Park et al., 2008), and this factor may be replaced by the histone deacetylase inhibitor, valproic acid (Huangfu et al., 2008a, b), emphasizing the important role for bHLH proteins in mediating chromatin remodeling. Finally, the class II bHLH protein, MASH-1, is fundamental to and the only transcription factor required for the trans-differentiation of primary adult fibroblasts into neuronal-like cells (Vierbuchen et al., 2010).

The last point of discussion concerns the use of GFP as an anterograde tracer to quantify axon regeneration in the current study. Although numerous GFP-positive brainstem axons were found to regenerate into the SC bridge, a direct comparison between treated and control animals was not possible because the control animals were infected with twice the amount of AAV-GFP. As discussed in Chapter 2, page 96, transduction units/mL of different AAV vectors, i.e. AAV-GFP and AAV-MASH-1, are not exactly the same. Nevertheless, in order for both control and treated animals to be injected with approximately the same number of infective AAV particles, the current study used less than a two-fold difference in the transduction units/mL of AAV-GFP and AAV-MASH-1. The control animals were injected with 19 µL of AAV-GFP and
the treated animals were injected with a total of 19 µL of AAV-GFP and AAV-MASH-1 in a 1:1 volume ratio. Thus, both groups received approximately the same number of infective AAV particles, but the control animals were injected with twice the amount of AAV-GFP.

Due to injection with twice the amount of AAV-GFP, control animals contained more GFP-labeled axons in the rostral spinal cord. In order to make a direct comparison between control and treated animals, the percentage of those axons rostrally that regenerated a given distance into the bridge was determined. However, based on the results from Chapter 2, Fig. 2.6e, that percentage would be confounded because, compared to the treated animals, the control animals were likely to exhibit a relatively large increase in the percentage of GFP-labeled regenerated axons at 1.0 mm or more into the bridge. Despite this, treated animals exhibited an increase in the percentage of GFP-labeled regenerated axons more proximally at 0.25 mm, 0.5 mm, and 1.0 mm, although this trend was not significant. This finding is consistent with the results from Chapter 2, Fig 2.6e. These results suggest that animals with different numbers of GFP-labeled axons in their rostral spinal cord do not exhibit a significant difference in the percentage of those axons found at 0.25 mm and 0.5 mm. Thus, at distances where differences in the amount of AAV-GFP may not produce a confounding effect, treated animals exhibited a trend towards increased brainstem axon regeneration. In conclusion, given that different amounts of AAV-GFP were used in control and treated animals, there is the possibility that MASH-1 expression also may enhance the regeneration of other brainstem axon populations.
The expression of MASH-1 in the brainstem enhances hindlimb joint movements after complete transection of the thoracic spinal cord and implantation of a SC bridge

The open-field hindlimb locomotion test, the BBB (Basso et al. 1995, 1996), was employed to compare MASH-1-treated and control animals on a weekly basis for six weeks following complete transection and implantation of a SC bridge. All the animals exhibited only a modest improvement and scores were confined to the lower end of the scale. In order to enhance the metric properties of the scale and assure the scores were continuous and ordinal, scores were converted from the 21 point scale to the modified 12 point scale (Ferguson et al., 2004). This modified 12 point scale, developed by two of the three originators of the 21 point BBB scale, does not require the animals to be re-analyzed and can be applied to any existing 21 point scale data set. In the current study, no animal received a score greater than 6 on the 12 point scale, which was equivalent to an 8 on the 21 point scale, indicating there was extensive movement of all three joints in both hindlimbs with plantar placement but no weight support. Using data from the 12 point scale, a two-way ANOVA revealed that MASH-1-treated animals exhibited significant improvement over the course of the six week study compared to controls. Furthermore, using either scale, 90 percent of the MASH-1-treated animals exhibited extensive movement of all three hindlimb joints at week six compared to only 45% of the controls, a result that produced a large Cohen's statistical effect size.

The significance of the scores at week six for MASH-1-treated animals is further confirmed when compared to scores from other studies of the complete
transection of the thoracic spinal cord, most of which were performed prior to 2004 and use the 21 point BBB scale. In the current study, MASH-1-treated animals averaged 7.4 and control animals averaged 6.2 on the 21 point BBB scale (p<0.05). Five additional groups of ten animals each (studied for other purposes) all received a SC bridge alone using the same surgical methods. Each of these 5 groups averaged between 6.1 and 6.3 on the BBB test after six weeks, analogous to the group injected with AAV-GFP alone in the current study. This large number of animals receiving the implantation of a SC bridge alone gives strength to the observed increase in the average BBB score for animals treated with MASH-1.

Historically, Basso et al. (1996) found that a complete transection of the thoracic cord followed by the implantation of gel foam only results in an average BBB score of less than 3 up to twelve weeks after injury. Even when combined with additional treatments, complete transection of the thoracic spinal cord does not typically result in week six BBB scores higher than 6.2 (Miura et al., 2000, Blits et al., 2003; Tsai et al., 2004, 2006; Fouad et al., 2005). Some studies of complete thoracic spinal cord transection, however, have found average six week BBB scores to be 8.2 after the implantation of a human SC bridge into the nude rat treated with methylprednisolone (Guest et al., 1997a), 7.5 after the implantation of both SCs and OEG together with housing the animals in a motor-enriched environment (Moon et al., 2006a), or 10 after the transplantation of human amniotic membrane with bone marrow stromal cells (Liang et al., 2009).
The error bars for MASH-1-treated and control animals tested at weeks three, four, and five, reveal variability (Fig 3.2i). This variability likely arises from animals that did not perform in a given week and thus received a zero, although they obtained high scores in the weeks before and after that timepoint. In order to account for such outliers and reduce variability, larger groups of animals may be used or animals may be tested more than once a week or for more than six weeks.

Whereas a total of 32 animals was BBB scored, only 21 of these animals were analyzed histologically due to the exclusion criteria listed in Chapter 2, page 82. These criteria were applied in a blind fashion. The most prevalent of these exclusion criteria was based on poor gross morphology of the SC bridge that was often compressed and sometimes did not contain any viable tissue. Interestingly, despite this poor morphology, a two-way ANOVA revealed that MASH-1-treated animals exhibited significant improvement in BBB scores over the course of the six week study compared to controls.

None of the animals in the current study obtained a BBB score higher than eight on the 21 point scale. This region of the scale represents hindlimb joint movements as well as crawling without weight support, and such behaviors are facilitated by central pattern generator (CPG) activity. Thus, CPG activity may have been primarily responsible for the observed improvements in hindlimb joint movements. In support of this, in the 21 animals analyzed histologically, there was no correlation between brainstem axon regeneration into a SC bridge and BBB scores. In addition, week one scores were included in the two-way ANOVA
analyses, which is a time period when brainstem axons are unlikely to have regenerated across the SC bridge. Together this suggests that although brainstem axon regeneration may be partially responsible for MASH-1-mediated improvements in hindlimb joint movements, components of the CPG are likely to be involved.

The regeneration of intraspinal axons may mediate the observed improvement in hindlimb joint movements. Intraspinal axons are well known to regenerate vigorously into a SC bridge (Xu et al., 1995a, 1997) and conduct action potentials (Pinzon et al., 2001). Given that cervicothoracic propriospinal neurons directly contribute to the CPG (reviewed by Jordan and Schmidt, 2002), in the present study the regeneration of these intraspinal axons into the SC bridge may have mediated the observed improvements in hindlimb joint movements. That supraspinal-mediated functional recovery through intraspinal relay neurons occurs has been reviewed by Bregman et al. (2002). In further support of this hypothesis, dendrites will grow in the presence of adult reactive astrocytes (Le Roux and Reh, 1996) and astrocytes extracted from scar tissue allow for the growth of dendrites (Rudge and Siler, 1990; McKeon et al., 1991). Such dendrites may be present at the caudal interface for synapse formation with intraspinal relay neurons. Thus, MASH-1 may have improved hindlimb joint movements through the modification of synaptic connections and/or arborizations between injured and/or uninjured brainstem neurons and their respective relay propriospinal neurons that have regenerated into the SC bridge. Follow up experiments to support this hypothesis might include using electrodes to
stimulate the spinal cord rostral to the bridge and record caudal to the bridge, followed by a subsequent transection of the bridge and an observed loss of behavior and/or post-synaptic potentials. Additionally, the injection of pseudorabies virus into the caudal spinal cord as a transneuronal tracer may elucidate a functional network that has been established across the site of complete transection.

Another mechanism by which MASH-1 may mediate improvements in hindlimb joint movements concerns the denervation, sprouting, and reorganization of spared spinal circuits after SCI that lead to significant molecular and morphological changes in the remaining spinal cord, as well as in the supraspinal centers (reviewed by Raineteau and Schwab, 2001; Edgerton and Roy, 2002; Edgerton et al., 2004). During the months and years after SCI, some functional recovery is observed in animal models (reviewed by Blight, 1993), as well as in patients, many of whom exhibit improvements on the ASIA scale (Waters et al., 1995; Burns et al., 1997; reviewed by Little et al., 1999). Following progressive Wallerian degeneration (Gillingwater et al., 2006) or denervation, the presence of “vacant” synapses results in extensive sprouting of spared axons as well as a strengthening of remaining synapses (reviewed by Dietz, 2010). Thus, the remaining spinal circuits no longer function in the same manner and are often hyper-excitble given the absence of supraspinal-mediated tonic inhibition (reviewed by Little et al., 1999). This leads to negative clinical manifestations such as hyperalgesia (Urban and Gebhart, 1999) and autonomic dysreflexia, but beneficial changes such as increased CPG activity. Thus, the
expression of MASH-1 prior to the complete transection may have primed neurons in the spinal cord caudal to the bridge for sprouting and/or synaptic reorganization, thereby improving CPG-mediated hindlimb joint movements. This hypothesis is supported by the observed improvement in MASH-1-treated animals despite exhibiting bridges with poor gross morphology and limited viable tissue.

In conclusion, this study demonstrates that expression of a developmental transcription factor is an intrinsic treatment strategy to enhance axon regeneration in the CNS. Furthermore, this study demonstrates that expression of a developmental transcription factor in the brainstem enhances hindlimb joint movements after SCI. Therefore, MASH-1 expression offers a promising building block on which to design intrinsic treatment strategies to promote axon regeneration and functional recovery.
Figures and legends

Figure 3.1: Stereotaxic injection of AAV-GFP plus AAV-MASH-1 leads to expression of MASH-1 in adult noradrenergic brainstem neurons. a) Immunostaining for DBH (yellow) reveals two noradrenergic neurons with Hoechst staining (blue) to visualize nuclei. b) Immunostaining for MASH-1 (red) shows the protein is expressed in the upper DBH-positive neuron. Careful analysis of immunostaining controls revealed that the red signal is from both MASH-1 protein and some bleed-through from GFP in the green channel. c) MASH-1 is only expressed in neurons that also expressed GFP (green). d) A merged image shows that the lower DBH-positive neuron is not infected with AAV and consequently does not express GFP or MASH-1 (scale bar=1 µm).
**Figure 3.2:** Animals injected with AAV-MASH-1 compared to controls exhibit increased regeneration of noradrenergic axons in the SC bridge and improved scores in an open field locomotion test, the BBB. a) In control animals, few DBH-positive noradrenergic axons (red, arrows) regenerate past the rostral spinal cord/SC bridge interface, delineated by GFAP immunostaining (white). Hoechst-stained nuclei are blue and asterisks indicate auto-fluorescent macrophages (scale bar=5 µm). b) In treated animals, many DBH-positive axons (arrows) regenerate past the rostral interface. Insets in panels a and b show low magnification images of the SC bridge; location of the high magnification images in either the same or adjacent section, is shown by a white box or letters. Arrowheads indicate the polymer channel (inset scale bar=1 mm). In treated animals many DBH-positive noradrenergic axons (arrows) regenerate 0.25 mm (c), 0.5 mm (d), 1.0 mm (e), and 2.5 mm (f). SCs expressing S100 are blue. Both the total number/mm² (g) and the percentage of DBH axons 10 mm rostral to the bridge (h) that regenerate into and across the bridge is greater in MASH-1 treated animals compared to controls (p<0.01 and p<0.001 respectively, two-way ANOVA; ** Bonferroni posttest p<0.01). i) MASH-1-treated animals demonstrated improved scores over all six weeks on the modified BBB scale compared to controls (p<0.05; two-way ANOVA; error bars=SEM). j) At week six, 90% of MASH-1-treated animals demonstrated extensive movement of all three hindlimb joints in both legs compared to only 45% of the controls (p<0.05; Chi-squared test) which was a large effect (d=0.97; Cohen’s test).
**Supplementary Figure 3.1:** MASH-1-treated animals exhibit GFP expression in noradrenergic axons that regenerate into a SC bridge.  

a-d) Expression of DBH (red) and GFP (green) is co-localized (arrows) in axons that regenerate past the rostral spinal cord/SC bridge interface, delineated by GFAP immunostaining in d (white; scale bar=5 µm).  

e-h) Expression of DBH (red) and GFP (green) is co-localized (arrows) in axons that regenerated in the SC bridge, identified by S100 immunostaining (blue; scale bar=5 µm). Arrowheads indicate the polymer channel. Inset in panel a shows a low magnification image of the SC bridge with the rostral spinal cord/SC bridge interface at the bottom left, delineated by white GFAP immunostaining with Hoechst staining in blue. Location of the high magnification images in an adjacent section is indicated by letters and small arrows. Arrowheads indicates the polymer channel (inset scale bar=1 mm).
**Supplementary Figure 3.2:** Animals injected with AAV-MASH-1 compared to controls exhibit improved scores in an open field locomotion test, the BBB. When comparing all animals subjected to the BBB, control (n=16) and MASH-1-treated animals (n=16) demonstrate improved scores over all six weeks on the modified BBB scale compared to controls (p<0.05; two-way ANOVA; error bars=SEM).
CHAPTER 4

ASSOCIATION OF GFAP-POSITIVE PROCESSES ENTERING A SCHWANN CELL BRIDGE WITH REGENERATION OF BRAINSTEM AXONS AND IMPROVEMENT IN HINDLIMB JOINT MOVEMENTS
Overview

Previous studies of complete thoracic spinal cord transection and implantation of a pre-gelled bridge of Schwann cells (SCs) and Matrigel found that intraspinal axons regenerate into the bridge, but additional treatments are required to promote axon regeneration from supraspinal neurons. However, the methods used in Chapter 2 enabled numerous brainstem axons to regenerate into a SC bridge without additional treatments. The present Chapter now addresses the nature of a SC bridge that is sufficient to promote the regeneration of brainstem axons without additional treatments. When the preparation of SC bridges for this dissertation work was slightly modified compared to previous work, by implanting an initially fluid rather than a pre-gelled bridge of SCs and Matrigel, the regeneration of brainstem axons was observed. In the initially fluid SC bridges, up to 12% of AAV-GFP-labeled brainstem axons rostral to the bridge regenerated 0.25 mm and 2% regenerated 2.5 mm across the bridge. In both pre-gelled and initially fluid bridges the rostral spinal cord/SC bridge interfaces varied from a sharp boundary to an irregular one where long glial fibrillary acidic protein (GFAP)-positive processes entered the bridge. However, the number of GFAP-positive processes that entered the SC bridge was greater in initially fluid bridges compared to those that were pre-gelled. Furthermore, initially fluid bridges exhibited a greater percentage of GFP-labeled brainstem and DBH-positive axons to regenerate at all measured distances when the bridges contained a greater number of GFAP-positive processes 0.25 mm beyond the rostral interface. The regenerating GFP-labeled brainstem axons and GFAP-
positive processes also appeared in close association with SCs, together forming a trio. Finally, improved scores for a hindlimb locomotion test, the BBB, also were associated with increased numbers of GFAP-positive processes that entered the bridge from both the rostral and caudal interfaces. In sum, the presence of GFAP-positive processes in a SC bridge defines a host spinal cord/SC bridge interface that is permissive for brainstem axon regeneration and improvements in hindlimb joint movements.

**Background**

Axon regeneration in the injured CNS environment is limited due to numerous deleterious and inhibitory substances, a multifaceted inflammatory response, and a deficiency of growth factors. This environment becomes progressively more inhibitory with the formation of the glial scar that serves as both a physical and chemical barrier to axonal growth (reviewed by Silver and Miller, 2004). At the foundation of the scar are dense sheets of basal lamina and a tight meshwork of reactive astrocyte processes (Feringa et al., 1979; Bernstein et al., 1985; reviewed by Reier et al., 1983). These structures resemble a multilayered version of the glia limitans, which together with the pial membrane, surround and contain the CNS. Incorporated within this glia limitans-like structure are additional components that impede axonal growth such as myelin debris, chondroitin sulphate proteoglycans (CSPGs), and semaphorins (reviewed by Sandvig et al., 2004, Busch and Silver, 2007). To surmount this environment, many extrinsic treatment strategies have been developed to increase axon regeneration, including the application of growth factors, antibodies against
components in myelin, and enzymes such as chondroitinase to degrade molecules present in the scar extracellular matrix (ECM; reviewed by Fawcett and Asher, 1999). In contrast to modifying the injured CNS environment, many groups have studied cellular and bio-matrix transplantation strategies to replace the lesion with a more permissive substrate for axonal growth (reviewed by Bunge, 2001, 2008; Tetzlaff et al., 2010).

Following upon early work by Ramon y Cajal’s student, Tello (1928), early CNS transplantation strategies were performed by implanting peripheral nerve grafts into complete transection injuries of the thoracic spinal cord (Kao et al., 1977a, b; Richardson et al., 1980). These peripheral nerve grafts in the thoracic spinal cord support intraspinal but not supraspinal axon regeneration (Richardson et al., 1982, 1984). Bunge (1975) proposed that SCs cultured from autologous peripheral nerve could be transplanted as a clinically relevant strategy. SCs implanted into demyelinated mouse spinal cord were found capable of remyelinating CNS axons (Duncan et al., 1981) and in combination with peripheral nerve grafts promoted axon regeneration after complete thoracic spinal cord transection (Wrathall et al., 1984).

More recently, polymer channels containing mixtures of SCs and Matrigel were implanted into the thoracic spinal cord after complete transection. Analogous to the peripheral nerve graft, these bridges supported the regeneration of intraspinal but not supraspinal axons (Xu et al., 1995a, 1997). In contrast, supraspinal axons did regenerate into SC bridges when additional treatments were introduced, such as the application of neurotrophins (Xu et al.,
methylprednisolone (Chen et al., 1996; Guest et al., 1997a); antibodies against inhibitory components in myelin (Guest et al., 1997b); implantation of olfactory ensheathing cells (OECs, Ramón-Cueto et al., 1998); or the use of chondroitinase (Fouad et al., 2005). Thus, after complete transection of the thoracic spinal cord, the implantation of SCs was previously demonstrated to support regeneration of intraspinal axons, but additional factors were required to entice supraspinal axons to cross the rostral host spinal cord/SC bridge interface and regenerate into the bridge.

The nature of astrocytes at the interfaces between transplants and host CNS tissue is critical for axon regeneration (reviewed by White and Jakeman, 2008). Astrogliosis and scar formation limit the regeneration of axons from the host spinal cord into grafts (Fishman et al., 1983) as well as the regeneration of axons out of grafts and back into the host spinal cord (Plant et al., 2001; Chau et al., 2004; Houlé et al., 2006; Tom and Houlé, 2008). Given the substantial proliferation of progenitor cells, the lesion environment is composed of a heterogeneous population of immature and mature astrocytes (reviewed by Hatten et al., 1991). Mature and reactive astrocytes are greatly inhibitory to axonal growth (Baehr and Bunge, 1990; Rudge et al., 1989; McKeon et al., 1991), especially when in contact with meningeal fibroblasts which promote the formation of glia limitans-like structures (Berry et al., 1983; Mathewson and Berry, 1985; Abnet et al., 1991; Franklin et al., 1992; Struckhoff, 1995; Hirsch and Bahr, 1999; Shear et al., 2003; Wanner et al., 2008). On the other hand, immature astrocytes promote axonal growth in vitro (Noble et al., 1994; Fallon et
The morphology of astrocytes appears to be indicative of their permissiveness for axonal growth. Axons are found to regenerate into CNS lesion environments in regions in which astrocytes exhibit linear alignment of their processes; these processes are often enclosed together with axons in a continuous basal lamina (Mathews et al., 1979c; Sims and Gilmore, 1983; Kawaja and Gage, 1991; Bunge et al., 1994; Davies et al., 1999; Tom et al., 2004). In contrast, few axons are found to regenerate when astrocyte processes rearranged in a tight meshwork that forms a sharp boundary enclosing the injury (Bernstein et al., 1985; Alonso and Privat, 1993).

A common feature of the early SC bridge studies was that the bridges were pre-formed by mixing the SCs with Matrigel and incubating them overnight prior to implantation, which allowed the SC-Matrigel mixture to gel inside the channel. Thus, when the bridge was implanted, there was the potential for fibroblasts, meningeal cells, and blood to enter the space between the host spinal cord and the pre-gelled SC graft if the implant did not conform to the surfaces of the spinal cord stumps. Such a cellular invasion could easily contribute to a glial scar that would impede the passage of regenerating axons (reviewed by Shearer and Fawcett 2001). In addition, in these early bridges, astrocyte processes were not observed to enter the SC bridge (Xu et al., 1995, 1997). However, when animals were implanted with SC bridges in combination
with the administration of methylprednisolone, some supraspinal axons regenerated and astrocyte processes were present in the bridge (Chen et al., 1996). Furthermore, Guest et al., (1997a, b) observed a highly interdigitated host spinal cord/SC bridge interface together with the regeneration of supraspinal axons in immunodeficient nude rats, which were also treated with methylprednisolone and/or antibodies that neutralize inhibitory components in myelin.

The present study employed a slight modification in bridge preparation, whereby the SCs were mixed with Matrigel seconds before implantation and the mixture was injected as a fluid into the polymer channel. This resulted in the regeneration of brainstem axons into and across the bridge, a significant difference from previous work utilizing pre-gelled bridges. The initially fluid bridges also contained highly irregular host spinal cord/SC bridge interfaces with numerous long GFAP-positive astrocyte processes entering the bridge. Furthermore, the number of astrocyte processes in the SC bridge was associated with the regeneration of brainstem axons as well as improvements in hindlimb joint movements. This is the first study to quantify astrocyte processes in an attempt to define a host CNS/graft interface as permissive or non-permissive for axon regeneration.

**Methods**

**Experimental design**

The experimental design is presented in Chapter 2, page 82. The only difference is that work in this Chapter used two separate groups of experimental
animals. In the first group, all animals (n=11) were implanted with an initially fluid SC bridge; whereas in the second group (n=14), seven of the animals were implanted with an initially fluid SC bridge and seven were implanted with a pre-gelled SC bridge. Otherwise, the experimental timeline and age of the animals in each experimental group was the same as described in Chapter 2.

**Generation of AAV vectors, stereotaxic injection of AAV, generation of purified SCs, and tissue processing and immunohistochemistry**

Details of these methods are presented in Chapter 2, pages 83-87.

**Spinal cord transection and initially fluid or pre-gelled SC bridge implantation**

The rats were anesthetized by intraperitoneal injection of 45 mg/kg ketamine and 5 mg/kg xylazine. A laminectomy was performed from thoracic vertebrae levels 7 (T7) to T9, with significant lateral exposure. Following dural removal from the exposed cord and severing of the dorsal roots, a single incision was made with angled micro-scissors to completely transect the spinal cord at T8. Then the ventral dura was severed and the ventral roots were cut. This procedure led to the retraction of the spinal cord stumps approximately 2-3 mm (Fig. 2.1c). Completeness of the injury was confirmed by lifting the rostral and caudal stumps. For the initially fluid bridges, a 5.0 mm long, polyacrylonitrile/polyvinylchloride (PAN/PVC) polymer channel, with a 2.7 mm inner diameter (kindly provided by Dr. P. Tresco, University of Utah, UT), was positioned in the gap, accommodating rostral and caudal stump insertion approximately 1 mm into each end of the channel. Then 3.0 X 10⁶ SCs in 15 µL DMEM were mixed with 10 µL of Matrigel (BD Biosciences, San Jose, CA) and
immediately injected through the rostral of two holes previously created in the top of the channel (Fig. 2.1d). For the pre-gelled bridges, $3.0 \times 10^6$ SCs in 15 µL DMEM were mixed with 10 µL of Matrigel and injected through one end of the same size polymer channel (without holes), and allowed to solidify for 20 minutes in the incubator before positioning in the transection gap. All SCs in this study were harvested from the same sciatic nerves and passaged at the same time. The SCs were kept on ice no more than 2 hours before implantation. The muscle layers and skin were closed with sutures and Michel clips, respectively. Following the surgery, the rats received subcutaneous injections of 5 mL physiological saline BID for seven days, gentamicin 5 mg/kg QD for seven days to prevent urinary tract infection, buprenorphine 0.1 mg/kg TID for three days, and their bladders were expressed manually BID until they regained micturition.

**Quantification of axon regeneration and GFAP-positive processes**

Adhering to guidelines established for the assessment of axon regeneration (Steward et al., 2003), sagittal sections of the SC bridge were analyzed by the line-transect method (Fig. 4.1a) and Neurolucida assisted computer microscopy. The exact length of the polymer channel was determined first to serve as a reference for the distance between the rostral and caudal zero points, $0'$ and $0''$, respectively. Next, the extent of host spinal cord tissue inside the channel was defined by the expression of GFAP-positive astrocyte somata, depicted in red at the spinal cord/SC bridge interface. The zero points, $0'$ and $0''$, were determined on the section that contained the tip of host spinal cord farthest from the rostral and caudal end of the polymer channel, respectively. This distance from the end
of the polymer channel was then used to locate 0’ and 0” on the remaining
sections. Then, dorso-ventral lines were drawn on each section along the rostro-
caudal axis at -10.0 mm, 0.25 mm, 0.5 mm, and 1.0 mm from 0’ as well as 0.25
mm, 0.5 mm, and 1.0 mm from 0”. To determine the dorso-ventral thickness of
the bridge tissue, the total length of Hoechst staining for each line was measured
inside the polymer channel. Then, the numbers of GFP positive axons (green
lines) and GFAP-positive processes (red lines) that cross a given dorso-ventral
line were determined, with the exception of the lines at 0’ and 0”. Thus, using the
distance and thickness of the tissue measured, all axon counts were determined
as either the number/mm² or the percentage of the labeled axons observed in the
rostral spinal cord, and GFAP-positive process counts were determined as the
number/mm².

Statistics

All statistical analyses were performed with the Graph Pad Prism 5
software. One-tailed nonparametric correlations with 95% confidence intervals
were performed on data obtained from 11 animals implanted with initially fluid
bridges. Two-way ANOVAs with Bonferroni post hoc analyses and one-tailed
Welch-corrected nonparametric t-tests with 95% confidence intervals were
performed on data from a second set of 14 animals, 7 of which received initially
fluid bridges and 7, pre-gelled bridges.
Results

Implantation of a SC bridge alone is sufficient for brainstem axons to regenerate after complete transection of the thoracic spinal cord

In all 25 animals, the complete transection of the thoracic spinal cord and implantation of a polymer channel containing pre-gelled or initially fluid SCs and Matrigel was sufficient for brainstem neurons to regenerate their axons. In animals that received initially fluid SC bridges, up to 12% of the GFP-labeled axons observed 10 mm rostral to the bridge were found to regenerate 0.25 mm into the bridge and up to 2% were found to regenerate 2.5 mm. Although carefully inserted into the polymer channel, six weeks after the implantation of the SC bridge the rostral and caudal spinal cord stumps were displaced varying distances inside the channel and occasionally up to 0.5 mm outside the channel. Furthermore, the spinal cord stumps were not always uniform in circumference and did not occupy the entire space within the channel walls. Despite these occurrences, regenerated brainstem axons were observed. The most regeneration was observed when the rostral stump occupied the majority of space within the channel walls and was inserted approximately 0.5 mm, as in Fig 4.1b.

In the initially fluid SC bridges, regenerated GFP-labeled axons were typically observed parallel to the longitudinal axis of the polymer channel and beyond GFAP-positive processes that entered the bridge from the rostral interface (Fig. 4.1b, c). The GFP-labeled axons were located in the periphery of the tissue bridge with the majority found ventrally (Fig. 4.1b, c). The population of regenerated brainstem axons appeared heterogeneous in that some axons
exhibited more robust GFP label than others and some appeared thinner and/or beaded (Fig. 4.1c, d). The GFP-labeled brainstem axons were in close proximity to SCs (Fig. 4.1d). Macrophages were also in the area of GFP-labeled axons (Fig. 4.1c) and in close proximity at the caudal interface (Fig. 4.1e). Many GFP-labeled brainstem axons regenerated across the rostral host spinal cord/SC bridge interface in regions where GFAP-positive processes entered the bridge (Fig. 4.1d). GFP-labeled brainstem axons regenerated across the entire bridge to reach the caudal SC bridge/host spinal cord interface (Fig. 4.1b, c, e). At the caudal interface, the GFP-labeled axons were no longer oriented parallel to the polymer channel, and were in close proximity to macrophages as well as astrocyte processes of the caudal spinal cord (Fig. 4.1e). In conclusion, the implantation of a SC bridge provides a permissive environment for some brainstem axons to regenerate after the complete transection of the thoracic spinal cord.

The presence of GFAP-positive processes is associated with increased regeneration of brainstem axons into a SC bridge

Upon examination of the host spinal cord/SC bridge interfaces of initially fluid SC bridges, distinct variations in the morphology of the GFAP-positive border were observed. In some interfaces, GFAP-positive somata and processes were positioned so as to form a sharp boundary (Fig. 4.2a); in other areas, however, the interface was highly irregular with many interdigitations of GFAP-positive processes (Fig. 4.2b). When the interface was sharply delineated by GFAP expression, GFP-labeled axons were not found to enter the SC bridge (Fig. 4.2a). At the irregular interfaces, GFAP-positive processes entered the
bridge and GFP-labeled axons entered the bridge as well (Fig. 4.2b, c). The GFAP-positive processes entered the bridge in a longitudinal orientation and extended parallel to the axons, both of which also were sometimes in close proximity to S100-positive/GFAP-negative SCs (Fig. 4.2b, c). Furthermore, these GFAP-positive processes were found to enter the bridge together with DBH-positive noradrenergic axons (Fig. 4.2d). Although GFAP-positive processes were found across the entire SC bridge, brainstem axons often were found to regenerate beyond the GFAP processes but remained in close proximity to SCs (Fig. 2b, c). Thus, the absence or presence of GFAP-positive processes entering the SC bridge appeared to determine whether the interface was non-permissive or permissive for brainstem axon regeneration into the bridge.

To examine this observation statistically, GFP-labeled axons and GFAP-positive processes were quantified using the line-transect method in a group of 11 animals that received the implantation of an initially fluid bridge. Animals were grouped into those with greater or fewer than 30 GFAP-positive processes/mm² found 0.25 mm past the rostral interface. Compared to animals with fewer than 30 GFAP-positive processes/mm², animals with greater than 30 GFAP-positive processes/mm² exhibited a striking increase in the percentage of regenerated GFP-labeled (Fig 4.3a; two-way ANOVA, p<0.001) and DBH-positive (Fig 4.3b; two-way ANOVA, p<0.005) axons at 0.25 mm, 0.5 mm, 1.0 mm, and 1.5 mm past the rostral interfaces. These animals also exhibited a direct correlation between the number of GFAP-positive processes/mm² found 0.25 mm past the rostral interface and the percentage of regenerated GFP-labeled as well as DBH-
positive axons at 0.25 mm, 0.5 mm, 1.0 mm, and 1.5 mm (data not shown). Together, these data demonstrate that the presence of GFAP-positive processes in a SC bridge is an important extrinsic factor for the regeneration of brainstem axons.

**Comparison of initially fluid with pre-gelled bridges of SCs and Matrigel**

Because this is the first investigation to report vigorous regeneration of brainstem axons following the complete transection of the thoracic spinal cord and implantation of rats SCs and Matrigel without any additional treatments, it is important to begin to understand the reasons for this finding. Early SC bridge studies had implanted a pre-gelled mixture of SCs and Matrigel. To determine if the observed axon regeneration in the present study was at least in part due to the novel implantation technique of injecting a fluid into the polymer channel, a second experimental group of 14 animals that received initially fluid bridges (n=7) was directly compared to animals that received pre-gelled bridges (n=7).

Immunohistochemical analysis of the host spinal cord/SC bridge interfaces for initially fluid and pre-gelled SC bridges revealed that in any given animal, both blunt and irregular regions of an interface could be found. However, most of the interface surface area of the pre-gelled bridges was usually sharp (Fig. 4.4a), whereas most of the surface area of the initially fluid bridges was irregular (Fig. 4.4b). In an attempt to quantify such differences, the line transect method illustrated in Fig. 4.1a, was employed for the analysis of GFAP-positive processes entering the SC bridge. At all measured distances from both the rostral and caudal interfaces, initially fluid bridges exhibited greater than a 10-fold
increase in the number of GFAP-positive processes entering the SC bridge compared to animals that received pre-gelled bridges (p<0.0001, two-way ANOVA, Fig. 4.4c).

The line-transect method of analysis was also employed to quantify the number of GFP-labeled axons that were observed to regenerate 0.25 mm, 0.5 mm, and 1.0 mm into the SC bridge. This analysis demonstrated that animals receiving the initially fluid bridges exhibited an increase in the amount of brainstem axons that regenerated to all measured distances compared to animals that received the pre-gelled bridges (p<0.001, two-way ANOVA, Fig. 4.4d). Animals that received initially fluid bridges exhibited an average of 229 GFP-labeled brainstem axons/mm² to regenerate 0.25 mm² into the SC bridge compared to an average of only 68 axons/mm² in animals that received pre-gelled bridges. Noteworthy is that the difference between the number of axons that regenerated into the bridge in animals that received initially fluid bridges versus animals that received pre-gelled bridges is greater at 0.25 mm compared to 1.0 mm (Fig. 4.4d). This suggests that the differences in the bridges may pertain more to the growth through the interface and into the bridge than growth across the bridge. In sum, the data demonstrate that compared to the pre-gelled SC bridges, the initially fluid bridges create improved interfaces for the regeneration of brainstem axons.

**Association of GFAP-positive processes and hindlimb locomotion test scores**

All of the animals (n=25) in this study were subjected to the open field hindlimb locomotion test, the BBB, on a weekly basis. Although all of the animals
in this study exhibited at least some improvement in hindlimb movements, none of the animals scored higher than an 8, and at most demonstrated extensive movement of all three joints in both hindlimbs, together with plantar placement and/or sweeping or crawling. In the experimental group of 11 animals that were implanted with an initially fluid SC bridge, the average weekly BBB scores were 1.2, 2.4, 4.0, 3.5, 5.3, and 6.1 respectively for the first six weeks after injury. This group of animals was grouped into those which exhibited a total of greater or fewer than 30 GFAP-positive processes/mm² at 0.25, 0.5, and 1.0 mm beyond both the rostral and caudal interfaces. Animals with greater than 30 GFAP-positive processes/mm² demonstrated increased week six BBB scores compared to those with fewer than 30 GFAP-positive processes/mm² (Fig 4.5) In addition, these animals exhibited a significant correlation between their week six BBB score and the total number of GFAP-positive processes/mm² at 0.25 mm, 0.5 mm, and 1.0 mm beyond both the rostral and caudal interfaces (data not shown). Thus, the number of GFAP-positive processes in a SC bridge is associated with improvements in hindlimb joint movements. However, BBB scores did not significantly differ between the experimental animals that received initially fluid (n=7) versus pre-gelled (n=7) SC bridges. Nevertheless, a strong trend at week four was observed for improved scores in animals that received initially fluid bridges compared to those that received pre-gelled bridges (Fig. 4.6).

**Discussion**

This study demonstrates that implantation of a SC bridge can be sufficient for brainstem axons to regenerate after complete transection of the thoracic
spinal cord, with up to 12% of labeled brainstem axons (found rostral to the bridge) regenerating into the bridge. This percent is equivalent to that of axons found to regenerate in the CNS after other treatment strategies (Park et al., 2008; reviewed by Goldberg, 2003). In addition, this study demonstrates that the observed regeneration of brainstem axons is associated with the number of GFAP-positive processes that enter the bridge from the rostral interface. Furthermore, improved hindlimb movements are also associated with the total number of GFAP-positive processes that enter the bridge at the rostral and caudal interfaces. Finally, this study demonstrates that, compared to a pre-gelled bridge, the implantation of an initially fluid bridge of SCs and Matrigel dramatically increased both the regeneration of brainstem axons and the number of GFAP-positive processes that enter the bridge. In sum, this is the first study to quantify the astroglial response as permissive or non-permissive by counting GFAP-positive processes in a SC bridge, and suggests that the nature of the interface between spinal cord and tissue transplant should be assessed carefully when evaluating treatment strategies designed to improve brainstem axon regeneration and hindlimb locomotion after spinal cord injury (SCI).

**Brainstem axons regenerate into a SC bridge without an additional treatment strategy**

A surprising observation from the current study was the regeneration of brainstem axons into a SC bridge without the use of additional treatment strategies. The regeneration was not limited to only specific populations, given the presence of axons with different morphologies and immunohistochemical properties. Previous studies found supraspinal axons to regenerate only when a
SC bridge was combined with additional treatments (Xu et al., 1995b; Chen et al., 1996; Guest et al., 1997a, b; Menei et al., 1998; Ramon-Cueto et al., 1998; Fouad et al., 2005). Thus, it was concluded that additional intrinsic or extrinsic factors were required to entice supraspinal axons to regenerate into SC grafts placed into sites of complete thoracic spinal cord transection.

One potential cause for the observed regeneration of brainstem axons into a SC bridge is that the stereotaxic injection of AAV may have produced an intrinsic conditioning lesion effect. A conditioning lesion of retinal and dorsal root ganglion cells (McQuarrie and Grafstein, 1981; Richardson and Issa, 1984) is well-known to enhance the regeneration of their axons into the CNS. The enhanced axon regeneration is thought to be mediated by growth and/or inflammatory factors released near the somata (Berry et al., 1996, 1999). Conditioning lesion effects have not been reported previously for brainstem neurons or the use of AAV. However, the infusion of 19 µL of AAV in the current study, albeit at a very slow rate, may have caused a release of inflammatory and/or growth factors near the brainstem somata, thereby priming some of the neurons to regenerate their axons into a SC bridge. This issue may be resolved by determining the number of retrogradely traced brain neurons that regenerate their axons into the SC bridge in animals that receive AAV-GFP, vehicle, or sham injections.

**Brainstem axons regenerate in the peripheral and ventral regions of the polymer channel**

As previously described for axon regeneration in SC bridges (Xu et al., 1995a, b, 1997; Chen et al., 1996; Guest et al., 1997a, b), the majority of axons
regenerated in the peripheral aspect of the tissue bridge and in particular the ventral region, where most of the S100-positive SCs are located. This suggests that SCs may settle at the bottom of the channel after transplantation, thereby increasing the regeneration of axons in that region. Cross-sectional analyses of the SC bridge in the previous studies reveal layers of fibroblasts around the exterior of the tissue bridges to form an epineurium-like structure, comparable to peripheral nerve (Xu et al., 1995a, 1997). The SC-related axons are found beneath this layer rather than more centrally, possibly due to oxygen requirements, although blood vessels do form in the tissue. The regenerated axons are situated in parallel along the longitudinal axis of the bridge. Their orientation may be due to alignment of SCs as in peripheral nerve and other matrices (Zhang and Yannas, 2005; Thompson and Buettner, 2006). The longitudinal nature of the polymer channel likely facilitates the fibroblast and SC-mediated formation of longitudinal basal lamina and collagen fibrils, which prevents the regenerating axons from crossing the polymer in a tangential or tortuous fashion.

**Brainstem axons regenerate in areas of a SC bridge that contain macrophages**

The regeneration of brainstem axons in the current study was often observed in areas occupied by macrophages. There has been a long debate as to whether macrophages promote, inhibit, or are simply permissive for axon regeneration (reviewed by Lotan and Schwartz, 1994; Donnelly and Popovich, 2008). Although macrophages have been found to promote axon regeneration in the CNS (Gensel et al., 2009), the interaction between CNS axons and ED1-
positive macrophages can initiate axonal dieback mediated by MMP-9 (McPhail et al., 2004; Horn et al., 2008; Busch et al., 2009). This interaction is dependent upon the state of the axon; dystrophic axons are subject to macrophage attack but neurons that receive a conditioning lesion to modify their intrinsic state are not (Busch et al., 2009). In the current study GFP-labeled axons that regenerate across the SC bridge and reach the caudal interface are in close association with macrophages. Therefore, the GFP-labeled axons may not be in a dystrophic state and/or the presence of SCs may somehow protect them from macrophage attack.

In the studies regarding macrophage-induced axonal dieback, SCs were not present, and their interaction with macrophages may have an important bearing on the growth of brainstem axons observed in the present study. In the PNS, SCs and macrophages have distinct interactions that promote the regeneration of axons mediated in part by cytokines (reviewed Martini et al., 2008; Cámara-Lemarroy et al., 2009). Infiltrating SCs express NGF after SCI (Krenz and Weaver, 2000; Brown et al., 2004), while both SCs and macrophages express NGF after peripheral nerve injury (Marcinkiewicz et al., 1999). This expression of NGF is mediated by autocrine and paracrine loops of IL-1 secretion by both macrophages and SCs (Lindholm et al., 1987; Shamash et al., 2002). Therefore, a similar cytokine-mediated feedback loop between SCs and macrophages may lead to increased expression of NGF in the bridge. This feedback loop may be amplified further by macrophage inflammatory protein-1 (MIP-1)-mediated proliferation of SCs (Khan and Wigley, 1994).
Several additional cytokine interactions between SCs, macrophages, and astrocytes may have a direct influence on scar formation and axon regeneration in the bridge. For example, TNF-α and IL-1β are expressed by SCs, macrophages, and astrocytes after SCI (Wagner and Myers, 1996; Shamash et al., 2002; Pineau and Lacroix, 2007). These cytokines induce autocrine- and paracrine-mediated expression of MMP-2, MMP-3, and MMP-9 by macrophages and SCs that together with TIMP-1 function to remodel basement membrane (La Fleur et al., 1996; Siebert et al., 2001; Shubayev et al., 2006; Chattopadhyay et al., 2007). Furthermore, axonal contact with SCs also induces them to secrete tPA (Clark et al., 1991). Together, the SC- and macrophage-induced expression of MMPs and tPA may open up channels in the scar to allow for axon regeneration.

Another cytokine that SCs express upon interaction with macrophages is IL-10, which reduces scarring in the PNS (Atkins et al., 2007) and may provide immunosuppression of macrophage-induced dieback through the inhibition of granulocyte-macrophage colony-stimulating factor (GM-CSF; Be’eri et al., 1998; Jander et al., 1996). In addition, IL-6 expression by macrophages and SCs after SCI (Leskovar et al., 2000; Pineau and Lacroix, 2007) may promote neurite growth (Hirota et al., 1996; Gölz et al., 2006) as well as axon regeneration (Wu and Bradshaw, 1996; Shuto et al., 2001). IL-6 also promotes neurotrophin expression by astrocytes and induces them to extend long processes in vitro (März et al., 1999). Together, the above mentioned autocrine and paracrine cytokine feedback loops between SCs, macrophages, and astrocytes may
remodel the ECM near the rostral spinal cord/SC bridge interface and thus enable the observed brainstem axon regeneration.

**GFAP-positive processes entering the SC bridge define an interface that is permissive for axonal regeneration**

Transplantation of a polymer channel containing SCs and Matrigel results in formation of host spinal cord/graft interfaces with variations between sharp and irregular GFAP-positive boundaries. Regions of the interfaces that were irregular exhibited long astrocyte processes that entered the bridge, sometimes up to 1 mm, whereas at sharp interfaces astrocyte processes formed a tight meshwork that did not extend into the bridge. The amount of brainstem axon regeneration directly correlated with the number of astrocyte processes that entered the bridge from the rostral host spinal cord/SC bridge interface.

The extension of astrocyte processes into sites of CNS lesions has been observed previously with or without axons. Following the complete transection of the spinal cord, axons sprout into the lesion site in association with astrocytes (Mathews et al., 1979b; Guth et al., 1981). The implantation of fibroblasts into the lesioned CNS also results in the extension of astrocyte processes, although axons do not enter unless neurotrophins are also applied to the lesion (Kawaja and Gage, 1991). This suggests that astrocyte processes do not require association with an axon in order to enter a lesion. In an analogous manner, astrocyte processes are found to cross the injured dorsal root entry zone (DREZ) without the regeneration of axons (reviewed by Fraher, 1999; Ramer et al., 2001a); axon regeneration is only observed with the application of additional factors such as neurotrophins (Ramer et al., 2000, 2001b; 2002; McPhail et al.,
chondroitinase and/or macrophage activation with zymosan (Steinmetz et al., 2005), a conditioning lesion (Richardson and Issa, 1984) or in combination with the local expression of PSA-NCAM (Zhang et al., 2007b, c). These studies suggest that the presence of astrocyte processes in the SC bridge may not require the presence of axons. Thus, the presence of astrocyte processes entering the site of a lesion may be the first step in creating a permissive environment for axon regeneration, but additional intrinsic and extrinsic factors are required to achieve the vigorous regeneration of axons. In the current study, the SC-mediated expression of growth factors may be the additional requirement that allows for brainstem axons to regenerate through a permissive astrocyte rostral host spinal cord/SC bridge interface.

Many groups have implanted grafts into spinal cord lesions and also observed the extension of astrocyte processes. For example, astrocytes processes have been found to enter grafts of collagen (Spilker et al., 2001; Yara et al., 2009), fibronectin (King et al., 2006), hydrogels (Woerly et al., 2001), and other biomatrices (Kataoka et al., 2004; Prang et al., 2006). In addition, astrocytes have been reported to enter grafts of OECs (reviewed by Li et al., 2005; Kocsis et al., 2009) and SCs transplanted into the spinal cord (Li and Raisman, 1997). However, in studies with pre-gelled bridges of SCs and Matrigel, astrocyte processes were not observed to enter the bridge (Xu et al., 1995a; 1997). In contrast, when the environment of the pre-gelled bridges was modified with growth factors (Xu et al., 1995b), or methylprednisolone (Chen et
al., 1996) in the nude, immunodeficient rat (Guest et al., 1997a, b), both astrocyte processes and supraspinal axons entered the bridge.

When minichannels containing SCs are implanted into the partially transected spinal cord, astrocytes processes as well as regenerated supraspinal axons are observed in the bridge (Xu et al., 1999), possibly caused by a reduced inflammatory response due to increased graft stability and/or duraplasty. This effect is dramatically enhanced when the minichannels containing SCs are transplanted in combination with GDNF (Deng et al., 2009). SCs, genetically engineered to express PSA-NCAM, implanted after SCI have improved ability to associate with astrocytes that extend processes into the transplant together with increased axon regeneration (Papastefanaki et al., 2007; Zhang et al., 2007a, b).

Although the studies mentioned above observed the extension of astrocyte processes into sites of lesions or transplants coincident with axon regeneration, astrocyte processes were only counted in one study. Kataoka et al. (2004) implanted bridges of alginate or collagen into sites of complete spinal cord transection and studied confocal images to count neurofilament-positive axons that were in association with astrocyte processes. The authors reported a difference in axon regeneration between groups but not the number of astrocyte processes associated with those axons. The authors also did not report total numbers of astrocyte processes and their quantifications were not dependent upon distance from the spinal cord/graft interface. The current study is the first to use the line-transect method to rigorously quantify the total number of GFAP-positive processes (as well as axons) at various distances from the host spinal
cord/graft interface and, consequently, demonstrate an association between the number of these processes and the number of regenerated axons. Given this association, future studies should elucidate the potential mechanisms by which astrocytes form permissive interfaces so that robust axon regeneration may be achieved.

Reactive astrocytes are often deleterious to axon regeneration and recovery of function, in contrast to supporting axon regeneration when in more immature-like or de-differentiated states (reviewed by Fitch and Silver, 1997a; Fawcett and Asher, 1999; White and Jakeman, 2008). After CNS injuries, including complete transection of the spinal cord, ependymal cells as well as mature reactive astrocytes may proliferate, express nestin, and give rise to immature astrocytes and/or radial glia (Mathews et al., 1979; Hunter and Hatten, 1995; Frisén et al., 1995b; Krum and Rosenstein, 1999; Leavitt et al., 1999; Mothe and Tator, 2005; Buffo et al., 2008; Busch et al., 2010). In the current study, some of the GFAP-positive astrocytes extending processes into the SC bridge exhibited a more bipolar morphology, similar to that of immature astrocytes. Thus, at the spinal cord/SC bridge interface, both ependymal cells and/or mature astrocytes could give rise to immature-like astrocytes that function to repopulate the lesion environment (reviewed by Gage et al., 1995b).

Radial glia and immature astrocytes are well-known to support axonal growth and regeneration (Singer et al., 1979; Silver and Rutishauser, 1984; Smith et al., 1986; Joosten and Gribnau, 1989; Silver et al., 1993; Pires-Neto et al., 1998; Iseda et al., 2004), which is likely accomplished by providing a
permissive environment, not unlike that during development. For example, the immature astrocytes at the lesion may express N-cadherin and growth-supportive integrins (Tomaselli et al., 1988). Noteworthy is that in addition to promoting axon growth, astrocyte expression of the β1 integrins mediates astrocyte polarity for migration as well as process extension (Etienne-Manneville and Hall, 2001; Peng et al., 2008). Bipolar NG2-positive progenitors, which give rise to immature astrocytes, support the regeneration of axons into the site of contusion and protect the axons from macrophage-induced dieback (Busch et al., 2010). Therefore, NG2-positive progenitors may be present in regions of the SC bridge where brainstem axons were observed near macrophages.

Given the potential to support axon growth, the direct transplantation of immature astrocytes was proposed as a treatment strategy for CNS injury by Silver (1988). They were subsequently found to reduce scar formation (Smith and Silver, 1988). Additional transplantation studies found immature astrocytes to migrate throughout the adult spinal cord (Goldberg and Bernstein, 1988) and promote modest improvement in axon regeneration after CNS injury (Kliot et al., 1990; Wunderlich et al., 1994). More recently, the transplantation of immature astrocytes into the acutely injured spinal cord has been shown to promote supraspinal axon regeneration (Joosten et al., 2004; Davies et al., 2006).

The GFAP-positive processes found in a SC bridge were longitudinally oriented and sometimes found in contact with S100-positive SCs and brainstem axons. These linear processes may provide paths for the axons to traverse scar tissue at the interface, analogous to studies of glial cell alignment in vitro.
(Deumens et al., 2004; Alexander et al., 2006; East et al., 2010). The description of astrocyte-like processes being associated with axons and SCs after CNS injury has been observed in electron micrographs by a number of investigators, including Mathews et al. (1979a), and Bunge et al. (1994). In the present study, brainstem axons were often found to regenerate beyond the GFAP-positive processes. This suggests that the GFAP-positive processes may be required to escort the brainstem axons across the interface, and that the SC bridge is sufficient to support axonal regeneration for longer distances.

**The implantation of an initially fluid bridge of SCs and Matrigel enhances axon regeneration compared to a pre-gelled bridge**

Direct comparison of initially fluid and pre-gelled SC bridges revealed that the use of initially fluid bridges leads to a dramatic increase in both brainstem regenerated axons and the number of astrocyte processes entering the bridge. Initially fluid grafts were first tested by Marchand and Woerly (1990) who reported the extension of astrocyte processes and regeneration of axons after grafting initially fluid type 1 collagen into transected spinal cords. Later, Joosten et al. (1995) performed a direct comparison of initially fluid and pre-gelled grafts of type I collagen and demonstrated astrocyte processes to enter together with a few corticospinal axons only when the graft was fluid.

The advantage of initially fluid bridges over pre-gelled bridges is likely due to several factors. First, in the pre-gelled SC bridges the major components of Matrigel, i.e. collagen type IV and laminin, may self-assemble into basal lamina-like structures, thereby contributing to or further instructing astrocytes to form glia limitans-like structures. Furthermore, axons may not be able to penetrate the
pre-gelled Matrigel without concurrent MMP activity (Pittman and Williams, 1989; Nordstrom et al., 1995). In addition, the initially fluid mixture spreads to rapidly conform to the host spinal cord stumps, which probably reduces the invasion of meningeal fibroblasts, inflammatory cells, and blood, analogous to the potential benefits of duraplasty limiting cellular invasion (Guest et al., 1997a, b).

Limiting the invasion of meningeal fibroblasts in the initially fluid bridges is likely to have significant inhibitory effects on scar formation at the host spinal cord/SC bridge interfaces. The invasion of meningeal cells and their interaction with astrocytes largely contribute to structures within the scar that resemble the glia limitans (reviewed by Hermanns et al., 2001; Shearer and Fawcett, 2001). This interaction also regulates the formation of the glia limitans during CNS development (Lyser, 1972; Kusaka et al., 1984; 1985; Pehlemann et al., 1985; Sievers et al., 1985). The role of meningeal cells in CNS/graft interfaces was previously described for fetal transplants (Kruger et al., 1986) where interfaces with multilayered glial processes and basal lamina contained meningeal cells, and regions without meningeal cells exhibited incomplete sheets of glial processes with no basal lamina.

In vitro, contact between astrocytes and meningeal fibroblasts results in their segregation from one another (Abnet et al., 1991; Franklin et al., 1992; Struckhoff, 1995; Hirsch and Bahr, 1999; Shear et al., 2003; Wanner et al., 2008), likely mediated by astrocyte expression of EphB2 and meningeal fibroblast expression of Ephrin-B2 (Bundesen et al., 2003). Despite this segregation, some astrocytes are reported to extend processes underneath the
meningeal cells in vitro (Struckhoff, 1995; Ness and David, 1997; Hirsch and Bahr, 1999; Shearer et al., 2003). Nevertheless, neurite outgrowth is only observed from meningeal cells onto astrocytes, not from astrocytes onto meningeal cells (Ness and David, 1997; Hirsch and Bahr, 1999). Neurites may be enticed to leave the growth-permissive astrocyte surfaces to extend onto fibroblasts, however, by the application of NG2-neutralizing antibodies and modifications to intrinsic states such as Rho or cAMP signaling (Shearer et al., 2003).

Studies of astrocyte-meningeal cell interactions, following injury to the DREZ (reviewed by Ramer et al., 2001a) or the transplantation of fibroblasts (Kawaja and Gage, 1991), demonstrate that astrocyte processes also have the potential to enter an interface that contains fibroblasts in vivo. This suggests that the reaction of astrocytes to a non-CNS environment, such as meningeal cells, may be dependent upon their internal state: more immature astrocytes might extend processes rather than forming a glia limitans. This is supported by in vivo work demonstrating that meningeal fibroblasts in scars do not segregate from immature astrocytes that express vimentin (Abnet et al., 1991). In conclusion, the above mentioned studies demonstrate that astrocytes have the potential to extend processes in the presence of fibroblasts, especially if astrocytes are in a more immature state. Thus, the reduced number of astrocyte processes in pre-gelled SC bridges may be, in part, mediated by a limited presence of immature-like astrocytes and/or the invasion of additional non-CNS cell types, such as leukocytes or erythrocytes. The invasion of these additional non-CNS cell types,
together with the fibroblasts, may then trigger astrocytes to create a glia limitans-like structure.

The interaction between astrocytes and meningeal cells is associated with increased expression of tenascins and CSPGs (Ajemian et al., 1994; Ness and David, 1997; Shearer et al., 2003; Tang et al., 2003). Although axons have been found to grow across some inhibitory components of the scar, such as CSPGs, in vivo they cannot cross the interface between the astrocytes and meningeal fibroblasts (Davies et al., 1999; Stichel et al., 1999a, b, c). Therefore, despite the potential for astrocytes to extend processes in the presence of fibroblasts, there may be increased expression of components that inhibit axonal growth. This suggests that additional intrinsic and/or extrinsic factors are required to induce supraspinal axon regeneration and overcome the presence of inhibitory factors.

In the current study, the presence of astrocyte processes may be the first step to promote supraspinal axon regeneration, with the presence of SCs providing additional factors. In this way, limited supraspinal axon regeneration may have been observed into pre-gelled bridges, despite the presence of SCs because the first requirement of astrocytic extensions was not achieved.

**GFAP-positive processes define a SC bridge as permissive for improvements in hindlimb movements**

The total number of GFAP-positive processes that entered the SC bridge from the rostral and caudal interfaces is associated with improvements in week six hindlimb joint movements. However, no animals obtained a BBB score higher than eight, suggesting that the changes in hindlimb movement may be, in part, mediated by central pattern generator (CPG) activity and not brainstem axon
regeneration (discussed in Chapter 3, pages 130-133). Analogous to the results from animals used in Chapter 3, there was no association between the amount of brainstem axon regeneration and BBB scores, suggesting that brainstem axon regeneration into the SC bridge does not mediate improved BBB scores up to a score of eight. Finally, the importance of the CPG for mediating BBB scores up to an eight is also supported by direct comparisons between animals that were implanted with initially fluid versus pre-gelled SC bridges. Comparison of BBB scores between these two groups did not reveal a significant difference, despite the fact that there was a significant increase in brainstem axon regeneration in animals that were implanted with initially fluid compared to pre-gelled SC bridges.
**Figures and legends**

**Figure 4.1:** The acute implantation of a polymer channel containing SCs and Matrigel is sufficient for brainstem axons to regenerate following complete transection of the thoracic spinal cord. a) Illustration of the line-transect method for quantification of GFP-labeled axons (green) and GFAP-positive processes (red) in a SC bridge. The polymer channel (thick black lines) and the transverse lines used for quantification (thin purple lines) are diagrammed. The numbers represent mm from the rostral (0') and caudal (0'') interfaces. Rostral cord left, caudal cord right. b) GFP-labeled brainstem axons (green) regenerate into and across the SC bridge surrounded by the polymer channel (arrowheads). The rostral spinal cord is indicated by GFAP (red) immunostaining and S100-positive SCs (blue) delineate the bridge. The white dashed box and letters indicate the region of high magnification images in c, d and e, respectfully. c) Numerous GFP-labeled brainstem axons (arrows) regenerate across the SC bridge. GFAP-positive processes (arrowheads) enter the bridge at the rostral interface. S100-positive SCs are blue. Asterisks indicate autofluorescent macrophages. The dashed line indicates the farthest extent of the host spinal cord, 0' (scale bars=250 μm). d) Many GFP-labeled brainstem axons (green) cross the rostral host spinal cord/SC bridge interface together with GFAP-positive processes (red) entering the bridge (arrowheads). S100-positive SCs are blue. e) GFP-labeled brainstem axons (green) regenerate across the entire SC bridge and reach the SC bridge/caudal host spinal cord interface as evidenced by GFAP immunostaining (red). Arrows indicate regions of close apposition between brainstem axons and astrocytes in the caudal spinal cord. Asterisks indicate autofluorescent macrophages (scale bars=10 μm).
Figure 4.2: Depending upon the interface, GFAP-positive processes cross the rostral host spinal cord/SC bridge interface together with brainstem axons. a) A non-permissive rostral interface, showing GFAP-positive astrocyte immunostaining (pink) forming a sharp boundary with S100-positive SCs (blue), where few GFP-labeled (green) axons enter the SC bridge. b, c) A permissive rostral interface showing an irregular boundary where GFAP-positive processes (pink) parallel with S100-positive SCs (blue) and many GFP-labeled (green) axons from the brainstem enter the SC bridge. Locations where GFP-labeled axons, GFAP-positive processes, and S100-positive SCs appear to overlap are yellow/white (arrows). Asterisks indicate autofluorescent macrophages. d) A permissive rostral interface, showing an irregular GFAP positive (blue) boundary where DBH-positive axons (red) and GFP-labeled axons (green) have regenerated into the SC bridge. Arrows indicate locations where GFAP-positive processes are in close association with DBH-positive axons (scale bars=20 µm). Insets with white dashed boxes indicate the regions on the same or adjacent sections of the rostral spinal cord/SC bridge interface where the higher magnification images were obtained. Arrowheads indicate the polymer channel (scale bar=200 µm).
Figure 4.3: Increased brainstem axon regeneration is observed when more GFAP-positive processes enter a SC bridge. Compared to animals with fewer than 30 GFAP-positive processes/mm² 0.25 mm beyond the rostral interface (n=4), animals with greater than 30 GFAP-positive processes/mm² (n=7) exhibit a greater percentage of their (a) GFP-labeled (two-way ANOVA, p<0.001; ** Bonferroni posttest p<0.01; * Bonferroni posttest p<0.05) and (b) DBH-positive (two-way ANOVA, p<0.005; *=Bonferroni posttest p<0.05) brainstem axons to regenerate at all measured distances.
Figure 4.4: Acute implantation of an initially fluid bridge of SCs and Matrigel instead of a pre-gelled bridge improves the host spinal cord/SC bridge interface for growth of axons into the bridge. a) A rostral interface with a pre-gelled SC bridge, showing GFAP-positive astrocyte immunostaining (pink) up to a sharp boundary with S100-positive SCs (blue), where few GFP-labeled (green) axons enter the SC bridge. b) A rostral interface with an initially fluid SC implant, showing an irregular boundary where GFAP-positive processes (pink) extend parallel with S100-positive SCs (blue) and many GFP-labeled (green) brainstem axons that regenerated into the SC bridge (scale bar=50 µm). c) The number of astrocyte processes that enter from both the rostral and caudal interfaces is greater in fluid versus pre-gelled SC bridges (two-way ANOVA, p<0.0001; **=Bonferroni posttest p<0.01; *=Bonferroni posttest p<0.05). d) The number of GFP-labeled brainstem axons that regenerate is greater into a fluid versus pre-gelled SC bridge (two-way ANOVA, p<0.001; *=Bonferroni posttest p<0.05).
**Figure 4.5:** Improvements in hindlimb joints movements are associated with increased numbers of GFAP-positive processes entering a SC bridge. The total number of GFAP-positive processes at 0.25 mm, 0.5 mm, and 1.0 mm beyond both the rostral and caudal interfaces was determined for each animal. Compared to animals with fewer than 30 total GFAP-positive processes/mm² (n=4), animals with greater than 30 GFAP-positive processes/mm² (n=7) exhibited improved scores at week six on the BBB test (non-parametric t-test, p<0.05).
Figure 4.6: Acute implantation of fluid SC/Matrigel does not improve hindlimb locomotion compared to a pre-gelled SC/Matrigel bridge. However, scores at week 4 approach statistical significance (p=0.053; non-parametric t-test; error bars=SEM).
CHAPTER 5

CURRENT AND FUTURE DIRECTIONS
Overview

In order to surmount the numerous obstacles that impede axons from regenerating after spinal cord injury (SCI), a single treatment strategy may not be sufficient but rather a combination of approaches that address both intrinsic and extrinsic factors may be required. Depending upon their mechanism of action, most therapeutics may need to be applied at distinct temporal periods after injury and during the recovery process. Although most current clinical strategies are designed to address the extrinsic factors, several promising new intrinsic pharmaceuticals may be advantageous. Gene therapy approaches also may become useful as more precise mechanisms of controlling gene expression are developed. With this technology the phenotypic state of neurons may be manipulated through tight regulation of transcription factor expression and chromatin-modifying agents. Intrinsic therapeutics are likely to be combined with extrinsic strategies such that the growth cone has a sufficient terrain upon which to regenerate. In addition, the intrinsic expression of some factors may have direct effects on the environment of the growth cone. Finally, as the wealth of information regarding axon regeneration evolves, new high-throughput translational injury models may be required to efficiently assess the enormous complexity of potentially synergistic treatments.

Current clinical treatment strategies

Most treatment strategies being tested clinically are designed for application during the early acute period, and therefore seek to reduce secondary damage, which will improve neural protection, reduce scar formation, and may
ultimately enhance axonal sprouting and regeneration. The design, performance, and analysis of clinical trials is critical (Blight and Tuszynski, 2006), especially since the desired level of axonal regeneration and recovery of function has not yet been observed experimentally. Common surgical interventions to reduce secondary damage after SCI are (1) decompression strategies that remove damaging bone, vertebral discs, and ligament fragments (2) stabilization of the spine, and (3) the application of shunts (reviewed by Gupta et al., 2010). There are also numerous pharmacotherapies for acute SCI that have been the subject of clinical trials.

The first clinical trial for acute SCI was the administration of methyprednisolone during the acute period after trauma in the National Acute Spinal Cord Injury Study (NASCIS; Bracken et al., 1984; Young, 1990). The rationale for using this glucocorticosteroid was to reduce the inflammatory response, edema, lipid peroxidation, axonal die-back, and the release of excitatory amino acids (reviewed by Hall and Braughler, 1982, and Ducker et al., 1994). With respect to the findings in this dissertation, methyprednisolone was previously found to induce the presence of astrocyte processes crossing a host spinal cord/Schwann cell bridge interface (Chen et al., 1996; Guest et al., 1997a, b). Nevertheless, despite extensive study on dosage and timing in subsequent clinical trials (Bracken et al., 1990; Bracken et al., 1997), there remains considerable controversy regarding the modest efficacy of methyprednisolone versus potential side effects in humans (reviewed by Becker et al., 2003; Fehlings and Baptiste, 2005; and Grupta et al., 2010). In addition to
methylprednisolone, the opiate antagonist, Naloxone, was tested in the second NASCIS trial and failed to produce therapeutic effects (Braken et al., 1990), although the rationale was based on observations that opioid peptides induce edema and hyperalgesia after SCI (reviewed by Long et al., 1986).

Thyrotropin-releasing hormone also was tested clinically due to its antagonistic effects on inflammation and endogenous opioids (reviewed by Long et al., 1986 and Faden et al., 1989). Although the results were promising in the relatively small study (Pitts et al., 1995; reviewed by Baptiste and Fehlings, 2006; and Grupta et al., 2010), no further studies have been reported. Another acute strategy tested in the clinic was the administration of the sialic acid-containing, glycosphingolipid, GM-1 ganglioside. Gangliosides are abundant in the outer membrane of neuronal cells in the central nervous system (CNS), and the administration of GM-1 after SCI was thought to reduce excitotoxicity and potentiate responsiveness to growth factors (reviewed by Skaper and Leon, 1992). However, the long-term clinical trial failed to produce significant improvements in motor function and GM-1 has not been approved for clinical use in the US (Geisler, et al., 2001, reviewed by Becker et al., 2003; Fehlings and Baptiste, 2005; Grupta et al., 2010). The NMDA receptor antagonist, gacyclidine, and the calcium channel blocker, nimodipine, have both been tested clinically in an attempt to reduce excitotoxicity after SCI, but neither one produced statistically significant effects (reviewed by Fehlings and Baptiste, 2005).

Finally, activated autologous macrophages were the first cells to be transplanted into patients with SCI in a phase 1 clinical trial (PROCORD). In this
trial, several of the patients recovered from the American Spinal Injury
Association (ASIA) scale grade A, with complete loss of both sensory and motor
function, to ASIA scale grade C, where they regained partial motor function
(reviewed by Rossignol et al., 2007; Hawryluk et al., 2008). This trial was
initiated based on reports that activated macrophages enhanced axon
regeneration after SCI (Rabchevsky and Streit, 1997), and promoted supra-
spinal axon regeneration and modest improvements in hind limb locomotion in
the transected rat spinal cord (Rapalino et al., 1998). Some of the beneficial
effects of activated macrophages are their reduced secretion of tumor necrosis
factor-alpha, as well as increased secretion of interleukin-1β (Fagan and Gage,
1990), brain-derived neurotrophic factor (Bomstein et al., 2003), interleukin-6
(Leskovar et al., 2000; Pineau and Lacroix, 2007), and oncomodulin (Yin et al.,
2006). Nevertheless, this trial was stopped because some patients exhibited
untoward effects.

Several promising therapies currently being tested in the clinic include
riluzole, minocycline, polyethylene glycol, Cethrin, and hypothermia. Riluzole, a
benzothiazole anticonvulsant, functions as a voltage-sensitive sodium channel
blocker to reduce the effects of excitotoxicity (Schwartz and Fehlings, 2001).
Minocycline, a tetracycline derivative, may have an anti-inflammatory effect of
reducing the macrophage response and thereby decreasing axonal dieback as
well as oligodendrocyte death (Wells et al., 2003; McPhail et al., 2004; Sterling et
al., 2004). Polyethylene glycol treatment is reported to enhance axon
regeneration after SCI through the re-sealing of injured axolemmas and the
inhibition of lipid peroxidation (Shi and Borgens, 1999, 2000; Shi et al., 1999, 2000; Luo and Shi, 2004; Luo et al., 2004). Cethrin, a Rho antagonist, is perhaps the most promising of the above mentioned pharmaceuticals and, as reviewed in Chapter one, pages 57-60, promotes axonal sprouting and regeneration through intracellular actions in the growth cone (McKerracher and Higuchi, 2006). Another promising therapy is hypothermia, which limits the secondary injury cascade through reductions in edema, hemorrhage, and inflammation (reviewed by Kwon et al., 2008; Dietrich, 2009). In support of this approach, early work used cold saline irrigation to cool the spinal cord during decompression surgery (Tator and Deecke, 1973). More recently, modest hypothermia achieved via an indwelling catheter has produced promising results in SCI patients (Levi et al., 2010). Lastly, based on the pioneering work of the Bunge laboratory and the Miami Project to Cure Paralysis, an investigational new drug (IND) application to the FDA is currently being prepared for the transplantation of autologous Schwann cells (SCs).

Rehabilitation is currently the gold standard treatment for chronic SCI patients and is fundamental to functional recovery. The rationale behind these programs is activity-induced muscle strengthening, axonal sprouting, and synaptic plasticity (reviewed by Sadowsky and McDonald, 2009; Van Hedel and Dietz, 2010). These programs are suggested to begin during the acute phase of injury, typically as an inpatient, and maintained when possible after hospitalization. Noteworthy is that rehabilitation programs are not simply limited to musculoskeletal training, but also may include cardiovascular, respiratory,
gastrointestinal, and urological regimes. Finally, in addition to rehabilitation, some pharmacological treatments for chronic SCI patients exist for spasticity as well as hyperalgesia (Cardenas and Felix, 2009; Teasell et al., 2010).

**Extrinsic treatment strategies**

Modifying the scar and creating a permissive host/transplant interface will likely be fundamental to the success of any sub-acute or chronic treatment regime. Strategies to achieve this include the use of enzymes to reduce inhibitory factors and restructure the extracellular matrix (ECM) in and around the lesion. With respect to inhibitory factors, the degradation of accumulating chondroitin sulphate proteoglycans (CSPGs) may be required for most combination strategies. Fortunately, this can be accomplished through an application of an enzyme, such as chondroitinase, to the scar and surrounding tissue (Bradbury et al., 2002; Houlé et al., 2006; Tom and Houlé, 2008). Restructuring the ECM also could involve the creation of tunnels, such as the bands of Bungner in peripheral nerve, those between fibroblasts and astrocytes at the dorsal root entry zone, or those created by olfactory ensheathing cells. The first step to form permissive tunnels through the ECM of the scar may require the degradation of dense collagen fibrils that accumulate in the injured spinal cord parenchyma. This may be achieved by the application of either matrix metalloprotease (MMP) -1,-3,-8,-13, or -14, all of which exhibit specificity for types I and III collagen. Once these fibrils are removed, the disintegration of type IV collagen in the dense basal lamina sheets may loosen the meshwork of astrocyte processes of the glia limitans, and this might be accomplished through
the application of MMP-2,-7, or -9 and/or concurrent inhibition of tissue inhibitors of metalloproteases. As holes are produced in the glia limitans-like structure that forms at the lesion site, astrocyte processes and axons might be able grow and create tunnels that are surrounded by a continuous basal lamina. From a practical stand-point, enzymes to restructure the ECM may be directly applied through local injections at the lesion site, however their half-lives might limit their efficacy. To surmount this, the use of viral vectors that carry transgenes for enzymes may induce their expression by endogenous cells around the lesion for longer durations, and thereby prove to be more useful than direct injections.

Based on the work in this dissertation, the presence of astrocyte processes may be required to establish tunnels through the lesion environment into which axons can grow. Therefore, the astrocytes adjacent to the scar may need to be changed into a more immature-like phenotype. This might be achieved pharmacologically through cytokines and growth factors, such as fibroblast growth factor and transforming growth factor (Cadwell et al., 2001; Sharif et al., 2007; Tripathi and McTigue, 2008; Dufour et al., 2009), or the use of genetic vectors to induce intrinsic modifications, such as the expression and/or repression of transcription factors and/or microRNAs (miR) that mediate astrocyte development. In particular, the transient expression of transcription factors such as Oct4, Sox2, and KLF4 (Park et al., 2008; Chang et al., 2009; Kim et al., 2009) and/or repression of miR-145 (Xu et al., 2009) may de-differentiate mature astrocytes into multi-potent cells. Given that multipotent cells transplanted into sites of SCI differentiate into astrocytes, if mature astrocytes
surrounding the lesion are transiently de-differentiated, they also are likely to re-differentiate into astrocytes, and during this process they may exhibit a more immature-like phenotype that facilitates axon regeneration.

The genetic modification of transplanted cells also may facilitate the formation of a permissive interface. These modifications may include the transient expression of neurotrophins, growth factors, cytokines, proteases, or transgenes that control the transplanted cells' phenotype. For example, SCs could be genetically modified to express the multi-neurotrophin, D15A, glial-derived neurotrophic factor, interleukin-6, poly-sialic acid linked neuronal cell adhesion molecule, as well as chondroitinase, all at specific timepoints after transplantation. In addition, the intrinsic manipulation of neurons themselves may have direct effects on the environment around the growth cone. In this way, the neuronal expression of transgenes such as MMPs or tissue plasminogen activator (Moon et al., 2006b), in combination with transplantation strategies may remodel the ECM locally around the growth cone. Local remodeling also may prevent the potential for hemorrhaging (due to loss of basal lamina around blood vessels) caused by previous work with enzymes (Feringa et al., 1979; Guth et al., 1980), and allow the regenerating axon to create a tunnel through the dense ECM of the scar. Finally, gene therapy-mediated control of neuronal phenotypes may induce them to increase their endogenous expression of MMPs.

**Intrinsic treatment strategies**

Some currently FDA-approved pharmaceuticals that need to be tested clinically with SCI patients include Accutane, Rolipram, and Valproic acid. Given
the role the retinoic acid receptor-β2 has on promoting axon regeneration (Yip et al., 2006; Wong et al., 2006), the cis-retinoic acid, Accutane, or an analogous pharmaceutical, also may serve as a useful intrinsic therapeutic. With respect to Rolipram, the numerous benefits of this type-4 phosphodiesterase inhibitor include intracellular elevation of cAMP-mediated reduction in scar formation, as well as increases in SC myelination, axonal regeneration, and functional recovery (Nikulina et al., 2004; Pearse et al., 2004). Furthermore, intracellular levels of cAMP act as a permissive switch to induce phenotypic changes through CREB and other downstream transcription factors that modify gene expression changes as well as chromatin structure. Thus, Rolipram may facilitate the binding of additional transcription factors that promote axonal growth and functional recovery. In an analogous fashion, the histone deacetylase, Valproic acid, may modify the chromatin so that phenotypic changes are more easily achieved in combination with vector-mediated expression of transcription factor transgenes.

The work from this dissertation demonstrates that gene therapy technologies may be useful to enhance the regeneration of axons after injury. However, before such strategies may be applied in the clinic, tight control of transgene expression and highly specific tropism of genetic vectors will likely be required. Then, potential genetic manipulations of germ cells that induce human-inheritable genetic modifications will be minimized, thus maintaining natural diversity in our genome. Currently, the cre-lox system of gene excision is perhaps the best and most efficient mechanism to turn off gene expression. In this system, transgenes of interest, flanked by the various lox elements, may be
excised upon the expression of a cre-recombinase, which efficiently and irreversibly silences their expression. Various other transgene regulatory strategies also exist, such as tamoxifen- and tetracycline-inducible systems, but they are often limited by “leaky” gene expression (reviewed by Sosa et al., 2009). As the nature of cis-regulatory regions and their respective factors become further elucidated, these regions may be applied to transgene cassettes of genetic vectors. Then complex cascades of transgene expression may be tightly controlled, analogous to gene expression patterns during development. Nevertheless, pharmaceutical technologies that manipulate chromatin-modifying proteins, and ultimately endogenous gene expression, may progress to the point that the introduction of transgenes is not even necessary to change a cells phenotype.

One of the primary goals of this dissertation work was to determine if the expression of a developmental transcription factor in mature CNS neurons would enhance the regeneration of their axons. Although, this work found that the expression a developmental transcription factor, MASH-1, produced a modest behavioral effect, the potential for transcription factors to promote axon regeneration and recovery of function may be enhanced by the use of additional intrinsic manipulations. Since the expression of only one regeneration associated gene (RAG) may not be sufficient to enhance axonal regeneration, and it is known that the expression of more than one such gene (GAP-43 and CAP-43) does promote axonal growth in the CNS (Bomze et al., 2001; Zhang et al., 2005), the co-expression of MASH-1 with additional transgenes may have a
synergistic effect. In particular, the co-expression of MASH-1 with additional transcription factors and/or transgenes that inhibit transcriptional repressors may facilitate the formation of a transcriptional node that regulates the expression of numerous RAGs.

Transcriptional nodes are complexes comprised of transcriptional machinery, co-factors, and chromatin-modifying proteins, which assemble upon binding to distinct cis-regulatory motifs and thereby mediate the expression of specific subsets of genes that determine the cell phenotype. Examples of such nodes were recently revealed in the cis-regulatory regions of RAGs in teleosts (Kusik et al., 2010) and in genes that specify photoreceptor phenotypes in mice (Hsiau et al., 2007). In addition, one of the most evolutionarily conserved transcriptional nodes utilizes an E-box motif and a cAMP- dependent CRE motif to mediate circadian patterns of gene expression (reviewed by Kato and Ishida, 1998; Hardin et al., 2004). The association of these two motifs is of particular interest given that cAMP modulators and CREB enhance the regeneration of axons in the CNS and bHLH proteins regulate neuronal states of differentiation. Therefore, the conserved combination of E-box and CRE motifs may, in part, mediate axonal growth as well. In line with this, the co-expression of MASH-1 with CREB enhances the responsiveness of 3T3 and PC12 cells to growth factors, by attracting CREB binding protein (p300/CBP) to the node, and thereby restructuring the chromatin (Mandolesi et al., 2002). Furthermore, the application of both cAMP and an upstream activator of MASH-1, bone-morphogenic protein-2, confers responsiveness of primary neural crest cultures to NGF, NT-3, and
GDNF (Reiriz et al., 2002). In addition to MASH-1, the paired homeobox (Phox2) proteins are required for cAMP-dependent transcriptional activation of noradrenergic differentiation (Swanson et al., 2000). Thus, when CREB, MASH-1, and Phox2b are expressed together, they may create a functional node that regulates axonal growth analogous to that which mediates noradrenergic differentiation (Pattyn et al., 2000). This is further supported by evidence that cis-regulatory elements of RAGs, such as GAP-43, contain coupled homeobox and E-box binding motifs (Fujimori et al., 2008; Kusik et al., 2010), and the NeuroD class II bHLH subfamily members, as well as GAP-43, both contain E-box and CRE motifs in their 5’ regulatory region (Bartholomae and Nave, 1994; Cho et al., 2001; Uittenbogaard and Chiaramello, 2002). Finally, KLF family members also may be important components of a node that changes the phenotype of mature neurons into one that is capable of axon regeneration. This is supported by the role KLF family members play in axon regeneration (Moore et al., 2009; Veldman et al., 2010), the presence of G/C rich regions in the cis-regulatory region of RAGs such as GAP-43 (Fujimori et al., 2008; Kusik et al., 2010), and the cooperative effects KLF and bHLH family members have in de-differentiating adult somatic cells (Park et al., 2008).

Although the expression of specific transcription factors will likely be required for axon regeneration, the simultaneous inhibition of repressors for those transcription factors may be equally important given. The bHLH and KLF family members which may repress axonal growth include the HES and Id subfamily members as well as KLF4 and KLF9, respectively (Konishi et al., 2004;
Lasorella et al., 2006; Moore et al., 2009). The inhibition of such repressors may enhance the ability of transcriptional activators such as MASH-1 or KLF6 to induce changes in gene expression. In addition to known repressors and activators, chromatin immuno-precipitation assays and reverse genetics approaches may reveal additional factors that complex with and regulate MASH-1, Phox2b, RAR-β2, or KLFs. Such assays also may identify additional cis-regulatory motifs for the expression of RAGs, and thereby elucidate additional factors required for a given phenotypic node. Finally, studies in the newly emerging field of miRs are elucidating their evolutionarily conserved roles in regulating phenotypic states during development and in the adult (reviewed by Kosik, 2009; Majer and Booth, 2010). Therefore, miRs are highly likely to regulate the expression of RAGs, and their manipulation may be fundamental to producing a neuronal phenotype that is capable of axon regeneration.

As reviewed in this dissertation, currently there are several genes/gene families that appear to be fundamental for axon regeneration, and these include PTEN, bHLH proteins, and KLF proteins. Therefore, these genes should be used as building blocks upon which to express additional transgenes and thereby produce synergistic effects on axon regeneration. With the wealth of potentially useful combinations of transgenes for intrinsic therapies to promote axon regeneration, more efficient and high-throughput injury models need to be developed. Towards this end, the brainbow transgenic mice offer an intriguing solution. Brainbow transgenic mice induce the combinatorial expression of fluorescent reporters through the stochastic recombination of various cre-lox
cassettes (Livet et al., 2007). By linking the expression of a given transgene, such as a transcription factor, to a given fluorescent reporter gene, after stochastic recombination, a given color of neuron will express a given combination of transgenes. In this way, numerous different transgene combinations may be analyzed in one animal, thereby creating within subject controls and a relatively high-throughput analysis of a given set of genes compared to individually testing one or two genes per animal.

With the continuing development of cellular transplantation, gene therapy, and pharmaceutical technologies, intrinsic and extrinsic treatments for axon regeneration will soon become a clinical reality. These tools will provide physicians with the potential to reverse the outcomes of SCI. Then, patients who suffer from such an affliction may soon begin to regain function and live long and fulfilling lives.
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