UNIVERSITY OF CALIFORNIA, SAN DIEGO

SnoN Facilitates Axonal Regeneration Following Spinal Cord Injury

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Jiun Lap Do

Committee in charge:

Professor Mark H. Tuszynski, Chair
Professor Armin Blesch
Professor Don Cleveland
Professor Binhai Zheng
Professor Yimin Zou

2011

The Dissertation of Jiun Lap Do is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2011

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

ACKNOWLEDGEMENTS

Chapter 2, in its entirety, is currently being prepared for submission for publication of the material. Do JL, Bonni A, Tuszynski MH. The dissertation author was the primary investigator and first author of this paper.

CURRICULUM VITAE

EDUCATION AND EXPERIENCE

2000 – 2004 University of California, Berkeley
B. A. in Molecular and Cell Biology – Neurobiology
Graduated with honor
Undergraduate research in the laboratory of Dr. Terry E. Machen
(8/2001 – 6/2004)

2004 – Present University of California, San Diego
Medical Scientist Training Program

2006 – 2011 Neurosciences Graduate Program, University of California, San Diego
Ph. D. in Neurosciences
Thesis advisor: Dr. Mark H. Tuszynski
Teaching assistant, *Basic Neurology*. Instructor: Dr. Mark Kritchevsky
(10/2006 – 12/2006)
Teaching assistant, Human Anatomy. Instructor: Dr. Mark Whitehead
(8/2007 – 12/2007)

AWARDS AND HONORS

2000	UC Berkeley Alumni Scholarship
2001	UC Berkeley Goldmann Scholarship
2000-2004	Robert C. Byrd Honors Scholarship
2004	Phi Beta Kappa Society
2004	Michael Kriegler Award

PUBLICATIONS AND MANUSCRIPTS

1. Tseng J, **Do J**, Widdicombe JH, Machen TE (2006). Innate immune responses of human tracheal epithelium to Pseudomonas aeruginosa flagellin, TNF-alpha, and IL-1beta. Am J Physiol Cell Physiol. 2006 Mar;290(3):C678-90.

2. Hybiske K, Fu Z, Schwarzer C, Tseng J, **Do J**, Huang N, Machen TE (2007). Effects of cystic fibrosis transmembrane conductance regulator and DeltaF508CFTR on inflammatory response, ER stress, and Ca2+ of airway epithelia.. Am J Physiol Lung Cell Mol Physiol. 2007 Nov;293(5):L1250-60.

ABSTRACT OF THE DISSERTATION

SnoN Facilitates Axonal Regeneration Following Spinal Cord Injury

by

Jiun Lap Do

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2011

Professor Mark H. Tuszynski, Chair

Functional recovery after spinal cord injury requires axons to overcome inhibitory factors, grow beyond the lesion site, and synapse on the appropriate target. We investigated whether developmental pathways necessary for axon differentiation and growth persist in the mature nervous system. We tested the hypothesis that manipulations of developmentally important neuronal TGF-β signaling would affect adult axonal regeneration following injury. We found that, in contrast to development, TGF-β inhibits growth of adult neurons and over-expression of SnoN, a developmentally regulated transcription factor that inhibits TGF-β signaling, rescues adult neurons from TGF-β

induced inhibition. More importantly, SnoN over-expression in sensory neurons was sufficient to enhance axon regeneration into a cell graft placed in the lesion cavity in an *in vivo* model of spinal cord injury.

CHAPTER 1

Introduction

Current State of Spinal Cord Injury Cases

Worldwide, 2.5 million individuals are estimated to suffer from some form of spinal cord injury (SCI) (International Campaign for Cures of Spinal Cord Injury Paralysis, www.campaignforcure.org). At the moment, it is an irreversible condition in which the numbers continue to increase by 130,000 new cases every year (International Campaign for Cures of Spinal Cord Injury Paralysis, www.campaignforcure.org). In the United States alone, 265,000 individuals are classified as SCI patients, 80.7% of which are male, and the number of new cases are expected in increase by 12,000 (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu). SCI has historically involved individuals with an average age of 28.7 years at the time of injury. However, more recent statistics report an average age of 40.7 years at the time of injury in the US (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu). Possible reasons for this shift may be reporting biases, increased survival rates of older individuals at scene of the accident, or the existence of a bimodal age distribution. While the number of SCI cases continues to increase, not all injuries present the same problems and deficits are contingent on the severity and type of injury.

The causes of SCI can be divided into compression, contusion, or penetration type injuries. Compression injuries result in the physical narrowing of the vertebral canal

resulting from fractures caused by traumatic forces. In spite of the implementation of safety protocols, such as seat belts and airbags that have reduced the incidence of spinal cord injury, automobile accidents continue to account for 40.4% of all spinal cord injuries in the United States (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu). Contusion types injuries, often resulting from falls (27.9%), are the second most common cause of SCI. The physical force of the fall is transduced to vulnerable neuronal cell bodies within the spinal cord and result in the loss of critical circuitry. Penetration type injuries often result from violence (15%%), primarily gunshot wounds. The end result of trauma to the spinal cord is determined by the extent and location of the primary injury and the physiological reactions that follow referred to as secondary damage.

Secondary Damage

In the hours following the primary insult, biological processes occur at the lesion site that extend the borders of the injury beyond its initial boundaries (Hagg & Oudega, 2006). These processes result in further loss of tissue, cystic cavitation, and a dying back or retraction of axons at the injury site. The mechanisms responsible for inducing secondary damage are not completely understood (Fehlings & Baptiste, 2005; Hurlbert, 2006; Rossignol *et al.*, 2007). Studies suggest immune cells may be partially responsible; macrophages and neutrophils infiltrate the normally immune privileged central nervous system (CNS) and release a number of molecules (Fitch *et al.*, 1999; Pearse *et al.*, 2003; Jones *et al.*, 2005). Free radicals are among these substance and interfere with electron

transport and cell metabolism, resulting in cell death (Saito *et al.*, 2005). Additionally, damage to neural structures cause excessive neurotransmitter release, leading to excitotoxicity and cell death (Park *et al.*, 2004). Furthermore, vascular disruption and decreases in respiratory output result in ischemia, hypoxia, and mechanical stresses from fluid extravasation that may lead to tissue necrosis (Chan, 2004; Hagg & Oudega, 2006). Such factors endanger tissue survival following SCI and exacerbate the functional outcome of the individual.

Classification of Injury Severity

The scale for assessing the severity of SCI is the American Spinal Injury Association (ASIA) Scale and range from grade A to E. ASIA grade A presents with the most severe deficits (loss of both sensory and motor function) and includes individuals with complete cord injuries. ASIA grades B to D present with less severe loss of function due to incomplete cord injuries and functional sparing. ASIA grade B retains a measure of sensory function but lacks motor function. ASIA grades C and D also preserves sensory function and retain a level of motor function that is either incapable (ASIA C) or capable (ASIA D) of movement against gravity. ASIA grade E designates cases in which function is indistinguishable from an uninjured individual.

While the requirements for ASIA classification are clearly defined, assessment of injury severity is difficult acutely following trauma due to spinal shock, a complete but transient loss of sensory and motor function caudal to lesion that can persist for hours to

weeks (Fawcett *et al.*, 2007). It is important to keep this caveat in mind when considering instances of functional improvement and conversion from initial ASIA assessments, especially within the first six months when recovery is most dramatic. While complete injuries such as ASIA A are less likely to spontaneously convert, 10-15% recover limited sensory function (ASIA B) and 3% recover limited motor function (ASIA grade D) (Stevens *et al.*, 2003; Fawcett *et al.*, 2007). In contrast, 54% of ASIA B cases recover motor capabilities to ASIA C or D and 86% of ASIA C and D patients regain limited mobility (Fawcett *et al.*, 2007). The greatest predictor of recovery following SCI is whether the injury was complete and the degree of spared functional tissue that remains. While the aforementioned rates of recovery do occur spontaneously, recovery is limited and inconsistent such that SCI patients frequently develop a number of sequelae and improvements in current therapies for SCI are necessary.

Clinical Complication and Treatments of Acute Spinal Cord Injury

The complications associated with SCI vary with the location and severity of the injury. Approximately half of all injuries involve the cervical spinal cord and can result in loss of upper and lower extremity function (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu). The trauma also affects a number of vital biological systems that increases the immediate danger an individual encounters.

During acute stages following SCI, complications with respiratory, cardiovascular, and embolic threats demand an emphasis on vital stabilization.

Respiratory complications may occur with cervical or thoracic lesions, requiring intubation if respiratory muscles are affected (Wuermser *et al.*, 2007). Cardiovascular issues resulting in bradycardia and hypotension limit essential perfusion of injured tissue (Furlan & Fehlings, 2008). Further, independent of SCI severity, embolic events such as deep vein thrombosis are emergent and life threatening (Merli *et al.*, 1993). These complications must be addressed prior to any medical or surgical intervention and also limit the early window in which therapies may be applied.

There is currently a lack of consensus on effective medical treatments for SCI, and surgical interventions are dependent on individual surgeon experience and expertise (Fehlings & Perrin, 2005; Hurlbert, 2006). Though one of the most common interventions targets the inflammatory system with the glucocorticoid methylprednisolone sodium succinate (MPSS), the scientific evidence surrounding its effectiveness is equivocal and difficult to interpret. The rationale fueling the use of MPSS was derived from the National Acute Spinal Cord Injury Study (NASCIS) II and III which concluded that patients who received treatment within eight hours demonstrated modest improvement in motor recovery; however, treatment with MPSS did not influence recovery of motor function one year after injury; however, (Bracken *et al.*, 1990; Bracken, 1991). This finding within a subgroup of participants propelled MPSS from clinical trials to what was once clinical standard and is now a reminder of the degree of criticism that must be exercised in pursuit of therapies for SCI.

A number of studies and observations following the initial finding called the effectiveness of MPSS into question. Primarily, MPSS had a narrow effective treatment

window within eight hours following injury based on a post hoc analysis of results that were overall not significant. Second, control subjects showed worse outcomes than had been historically published (less improvement) (Bracken *et al.*, 1992; Hurlbert, 2006). Further, unassociated trials did not find a significant MPSS effect and, in some cases, observed greater deficits and increased risk of death (Roberts *et al.*, 2004; Hurlbert, 2006). Additionally, prolonged methylprednisolone treatment increased the incidence of adverse effects such as severe sepsis and pneumonia (Bracken *et al.*, 1997). Perhaps the most damning evidence against the effectiveness of MPSS is the finding that the suggested MPSS doses may cause acute corticosteroid myopathy and reduce initial assessments of motor function (Qian *et al.*, 2005). Therefore, the recovery to MPSS treatment is in fact recovery from MPSS induced myopathy.

Surgical interventions for SCI lack the standardizations found for other conditions. Surgical options often include closed reductions, spinal stabilization, or decompression. There is no standard for surgical treatment and the timing of these operations is not well established (Fehlings & Perrin, 2005). Animal studies suggest that intervention within eight hours is beneficial (Dimar *et al.*, 1999; Carlson *et al.*, 2003) while a meta-analysis concluded that early (within 24 hours) decompression improved outcomes (La Rosa *et al.*, 2004). However, early interventions carry risks of surgical complications and present practical concerns of accessing properly equipped facilities. In contrast, trials that randomly assigned patients to early (within 72 hours) or late (more than five days) surgery found no significant improvement from early surgery (Vaccaro *et al.*, 1997). While early intervention fell outside the 8-24 hour window in these cases, 72

hours represents a more practical period in which vitals can be stabilized and the patient transported to the necessary facility. These discrepancies in outcomes and logistical difficulties complicate the development of standardized treatment and demand consideration when designing novel therapies for acute SCI.

Clinical Complications with Chronic SCI

Death following admission for acute traumatic SCI ranges from 4 to 20 percent (Daverat *et al.*, 1989; Claxton *et al.*, 1998). A relatively low mortality rate means that survivors will likely face years with limited functional improvement and additional medical complications encompassing a number of biological systems: respiratory, cardiovascular, urinary, gastrointestinal, reproductive, and musculoskeletal. The primary cause of death among chronic SCI patients are respiratory illnesses (21.2%), infections and parasitic diseases (11.1%), and heart disease (9.5%) (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu).

The loss of respiratory muscle innervation and need for ventilator assisted respiration can be one of the most visible and disabling complications of SCI. However, chronic SCI patients are also at greater risk for pneumonia due to impaired cough reflexes and clearance of secretions (McKinley *et al.*, 1999). Pneumonia accounts for 69.7% of all respiratory related deaths among SCI patients (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu).

Cardiovascular complications are also an emergent concerns. Loss of descending autonomic inputs can result in autonomic dysreflexia, a biological phenomenon in which sympathetic stimulation results in vasoconstriction and hypertension for which parasympathetic induced bradycardia is unable compensate (Bycroft *et al.*, 2005). The resulting elevated blood pressure can lead to cardiac arrest, seizures, and intracranial hemorrhage. Chronic SCI patients are also at higher risk for coronary artery disease. Risk factors such as elevated lipids and glucose are more prevalent following SCI and increase the prevalence of coronary artery disease by three to 10 fold compared to the general population (Myers *et al.*, 2007).

Gastrointestinal and genitourinary systems are also affected by SCI. A neurogenic bladder develops from loss of coordination between descending supraspinal and sacral micturition centers. Dysregulation can potentially lead to a bladder that has hyperactivity, impaired emptying, elevated pressures, urinary reflux, or urinary retention. Without proper management, these complications can triggers for autonomic dysreflexia and result in renal function decline (Karlsson, 1999). Urine retention also increases the probability of urinary calculi and urinary tract infections, potential antecedents to life-threatening septicemia (McKinley *et al.*, 1999; Siroky, 2002). Gastrointestinal complications commonly require proper evacuation of impacted colons and adversely affect quality of life (Stone *et al.*, 1990; Glickman & Kamm, 1996).

As a consequence of limited mobility, musculoskeletal and dermatological health are also affected. In the absence of load bearing and resistance, osteoporosis develops in bones below the injury, increasing the risks of fracture (Lazo *et al.*, 2001; Jiang *et al.*,

2006). Paradoxically, chronic SCI patients may also develop heterotopic ossification, bony deposits in tissue surrounding peripheral joints, that decrease range of motion and sources of joint inflammation (van Kuijk *et al.*, 2002; Teasell *et al.*, 2010). Immobility and spasticity can lead to muscle contractures, a shortening of muscle fibers relieved only by orthopedic intervention (Dalyan *et al.*, 1998; Harvey & Herbert, 2002). Pressure ulcers of the skin are also a consequence of immobility that can lead to infection and septicemia (Yarkony, 1994; Chen *et al.*, 1999).

The collection of these complications grossly affects quality of life. With proper treatment and vigilance in preventative measures, the risks are reduced but remain significantly increased compared to the normal population. These complications take not only physical tolls but also psychosocial and economic ones.

Psychosocial and Economic Consequences

The new spinal cord injury patient faces a drastically altered life and a number of psychological hurdles. Depression is common and symptoms most likely appear within the first month after trauma regardless of injury severity. The frequency of depression in SCI populations is estimated to be five times that of the general population with a prevalence of 20-45% of patients reporting depression following trauma (Kirshblum *et al.*, 2007). As a correlate, risks of suicide is also increased by two- to six-fold compared to the general population (DeVivo *et al.*, 1991). Suicide is the leading cause of death in

SCI populations younger than 55 years and is more likely to occur within the first 5 years (Kirshblum *et al.*, 2007).

Compounding the psychiatric issues are also economic concerns. The direct cost of care for spinal cord injury is estimated to be approximately $1 million dollars for the first year of disability and about $171 thousand dollars for each subsequent year in high cervical injuries (National Spinal Cord Injury Statistical Center, www.nscisc.uab.edu). It is estimated that the United States alone will spend $7.7 billion dollars on care and social welfare (International Campaign for Cures of Spinal Cord Injury Paralysis, www.campaignforcure.org). Potentially, the actual cost of SCI is even greater as these estimations do not account for indirect costs such as loss of wages, benefits, and productivity.

Furthermore, SCI, like other disorders, affects more than just the individual. It is estimated that 40-45% of individuals with SCI require some type of personal assistance (www.spinalcord.uab.edu). It is often the family members and unpaid care givers who satisfy this need. The contributions and sacrifices of these individuals should also be considered when estimating the cost of SCI and should not be neglected. Therefore, when treating disease, one treats the individual and their community.

Neuron-Extrinsic and -Intrinsic Factors Affecting Regeneration

Following the 1981 work of David and Aguayo in which it was unequivocally demonstrated that CNS axon were capable of regenerating into a peripheral nervous

system (PNS) graft, it was common to consider factors limiting regeneration as extrinsic or intrinsic. Extrinsic referred to the environment surrounding the neuron while intrinsic referenced intraneuronal molecules and processes. This is a classification that is not entirely true. Neurons express receptors that transduce extrinsic signals into intracellular cascades. Conversely, neurons are capable of producing and secreting molecules and compounds that affect extracellular characteristics or altering receptor expression to change responses to extrinsic cues. However, this historical framework continues to be relevant as it has been the bases for a number of important studies that constitute the foundation for a therapy to SCI which will involve a combination of approaches.

Extrinsic Factors Affecting Regeneration

The environment external to the neuron is drastically altered following injury but also endogenously expresses factors inhibitory for axonal growth. Numerous studies indicate that CNS myelin inhibits axonal growth and is composed of a number of inhibitory molecules (Schwab & Bartholdi, 1996; Filbin, 2003). In addition to these endogenously expressed molecules, injury to the spinal cord drastically alters the environment and induces an influx of inflammatory cells, upregulated inhibitory extracellular molecules, activation of glial cells, a loss of cellular substrate for growth, and a loss of trophic support (Silver & Miller, 2004; Ramer *et al.*, 2005). A number of studies have attempted to address these inhibitory factors through the reductions of inflammatory infiltrations, neutralization of inhibitory elements, degradation of the extracellular matrix (ECM) , inhibition of the glial scar, reconstitution of lost cellular

substrate, and replacement of neurotrophic factors. The contributions of these factors to regenerative failure and the successes and concerns of approaches intended to attenuate their effects will be addressed in the following sections.

Extrinsic Factors Affecting Regeneration: Inflammation

The role of microglia in regeneration is not well established. Microglial cells are resident immune cells intrinsic to the central nervous system and readily respond to injury. Microglia are capable of releasing of cytokines that recruit leukocytes and macrophages to the injury site and have demonstrated limited degrees of phagocytosis (Thanos, 1992). Activated microglia release cytokines such as TNF-α and free radicals that can be toxic to neurons and further exacerbate cell loss as a primary mediator of secondary damage (Banati *et al.*, 1993; Giulian, 1993). Neutralizing cytotoxic components significantly reduces the degree of cystic cavitation and secondary damage (Fitch *et al.*, 1999). Suppressing microglial activation with minocycline or tetracycline has been shown to have a neuroprotective effect on retinal ganglion cells (Baptiste *et al.*, 2005). However, contrasting studies have demonstrated microglia capable of secreting a number of trophic factors and suggest that activated microglia may be beneficial following trauma (Rabchevsky & Streit, 1997).

Macrophages emulate a similar role as microglia in the PNS; similarly, the role of macrophages in regeneration is just as convoluted. In some circles, macrophages are considered critical for regeneration. The PNS possesses a capacity for growth beyond

what is observed in the CNS, a property that may be explained by a greater efficiency in clearing myelin debris following injury in the PNS. Macrophages directly contribute to this enhanced efficiency (Beuche & Friede, 1984; Perry *et al.*, 1987). Macrophages are also capable of secreting trophic substances and antioxidants that may promote neuronal survival and regeneration of injured axons (Baichwal *et al.*, 1988; Elkabes *et al.*, 1996). Studies utilizing zymosan, a lipopolysaccharide that activates macrophages, generate greater traction for the idea that macrophages stimulate growth and regeneration. In retinal ganglion cells (RGCs), zymosan injections into the vitreous promoted regeneration of injured optic nerves (Leon *et al.*, 2000). Oncomodulin, a calcium binding protein, was identified as the macrophage-derived molecule induced by zymosan stimulation responsible for promoting RGC regeneration (Yin *et al.*, 2006). The combination of oncomodulin and cyclic adenosine monophosphate (cAMP) was found to significantly enhance retinal ganglion cell regeneration (Yin *et al.*, 2003; Yin *et al.*, 2006). Furthermore, the transplantation of macrophages activated by zymosan promoted the regeneration of injured somatosensory neurons (Lazarov-Spiegler *et al.*, 1996; Steinmetz *et al.*, 2005).

The body of work suggesting that inflammatory cells are beneficial for regeneration is directly contradicted by studies demonstrating a detrimental effect from inflammation. Zymosan-induced inflammation *in vivo* results in progressive cavitation and secondary damage (Fitch *et al.*, 1999). Furthermore, macrophages directly interact with neurons and inhibit growth. When the axons encounters an inhibitory matrix, the growth cone becomes dystrophic and activated macrophages physically interact with

dystrophic growth cones and induce retraction (Horn *et al.*, 2008). Additionally, macrophages have also been shown to be capable of TNF-α release, a cytokine with inhibitory effects on neurite growth and neuronal survival (Selmaj & Raine, 1988; Yune *et al.*, 2003).

Extrinsic Factors Affecting Regeneration: Glial Scar

Another set of cellular events affecting regeneration involve glial cells and the formation of a characteristic glial scar. This primarily involves astrocytes, which are resident to the spinal cord and normally serve a supportive role for neurons. Following injury, astrocytes become reactive and undergo hypertrophy and proliferation characterized by increases in glial fibrillary acid protein (GFAP) immunoreactivity (Sofroniew, 2005). GFAP-immunoreactive astrocytes persist at the lesion borders long after injury and demarcate lesion boundaries.

Overall, studies demonstrate an inhibitory role for GFAP-immunoreactive astrocytes populating the lesion border. However, the formation of a glial scar preserves tissue and prevents secondary damage as selective ablation of astrocytes following injury resulted in greater degrees of functional deficit and tissue loss (Faulkner *et al.*, 2004). To some extent, certain types of astrocytes are beneficial and may support axon growth through the production of laminin (Liesi *et al.*, 1984; Grimpe & Silver, 2002). CNS axons do associate with GFAP processes that extend into the lesion site when a cellular

substrate and growth factor support is provided (Kawaja & Gage, 1991). However, this growth is not extensive and may indicate presence of overriding inhibitory mechanisms.

The activation of astrocytes coincides with the deposition of a number of ECM molecules that create a physical barrier to regeneration (Silver & Miller, 2004). Additionally, reactive astrocytes are capable of secreting diffusible factors that inhibit growth (Smith-Thomas *et al.*, 1994) and cytotoxic molecules such as nitric oxide and proinflammatory molecules (Antony *et al.*, 2004; Ikeda & Murase, 2004). However, astrocytic contributions to the ECM are the major contributors to inhibition. Suppressing formation of the glial scar and ECM deposition using taxol has been correlated with increased axon regeneration and functional improvements (Hellal *et al.*, 2011; Sengottuvel *et al.*, 2011). Tenascin is a major molecule produced by astrocytes with profound effects on axon growth (McKeon *et al.*, 1991).

One of the most well characterized of ECM molecules affecting axon growth are those belonging to the family chondroitin sulfate proteoglycans (CSPG). CSPGs consist of a protein core linked to glycosaminoglycan (GAG) side chains and include neurocan, versican, brevican, phosphocan, and NG2 (Yamaguchi, 2000). Developmentally, CSPGs play a role in axon guidance (Bandtlow & Zimmermann, 2000; Bovolenta & Fernaud-Espinosa, 2000). CSPGs effectively inhibit neurite outgrowth *in vitro* (Snow *et al.*, 1990; McKeon *et al.*, 1991; Snow *et al.*, 1991). Inhibition is derived from the expression of the GAG chains and astrocytes known to inhibit growth express CSPGs (McKeon *et al.*, 1995). Ezymatic removal of the GAG chains from CSPGs is possible using chondroitinase ABC. GAG chain degradation is sufficient to reduce its inhibitory effect

on neurite outgrowth *in vitro* (McKeon *et al.*, 1991; McKeon *et al.*, 1995). GAG chain removal *in vivo* is also sufficient to promote growth of lesioned axons into CSPG released areas and improve behavioral and electrophysiological outcomes (Moon *et al.*, 2001; Bradbury *et al.*, 2002; Houle *et al.*, 2006a). Behavioral and anatomical benefits can be greatly enhanced with physical therapy or training. However, this therapeutic window is limited and the behavioral improvement is at the expense of other unused system (Garcia-Alias *et al.*, 2009).

Extrinsic Factors Affecting Regeneration: Loss of Cellular Substrate

The loss of a physical substrate on which to grow is another obstacle that must be overcome in order to promote regeneration. Initial tissue loss results from the primary insult to the CNS. Secondary damage follows as a consequence of ischemia, hypoxia, edema, excitotoxicity, inflammation, necrosis, or apoptosis. Cystic cavitation does not always follow SCI but is common in humans, nonhuman primates, and some mammalian models of spinal cord injury (Ma *et al.*, 2001; Moon & Bunge, 2005). Investigations have attempted to address this loss of tissue with a variety of strategies incorporating tissue grafts, cell grafts, and biomaterials.

Ramon y Cajal initially described growth of injured CNS axons as abortive and impossible (Ramón y Cajal *et al.*, 1991). It was not until the 1980's when David and Aguayo grafted peripheral nerves and observed regeneration of select CNS axons using modern tracing techniques that growth of injured CNS became a possibility (David &

Aguayo, 1981). Studies that followed using predegenerated peripheral nerves demonstrated that ascending sensory axons, raphespinal, and reticulospinal axons regenerate (Oudega *et al.*, 1994; Hiebert *et al.*, 2002). While this approach can yield significant results, logistical issues prevent broad spectrum adaptation. Primarily, peripheral nerve grafts are acquired from an autologous source, the sural nerve, which requires surgical interventions that are not without complications and results in a degree of sensory loss in the foot. It can also be difficult to acquire sufficient amounts to treat a large injury. Additionally, axon growth is from a limited populations of CNS tracts that do not include corticospinal tract (CST) axons and axons that enter this bridge continue to encounter an inhibitory CNS environment upon emergence (Levi *et al.*, 2002). Furthermore, successful grafting requires resection of the lesion site which may further exacerbate the injured condition.

Peripheral nerves are primarily composed of Schwann cells, myelinating cells of the peripheral nervous system; this characteristic put forth the hypothesis that Schwann cells produce a permissive substrate for growth. Schwann cells are, in fact, capable of secreting trophic factors and are often found to migrate into a lesion site following injury (Bunge, 1994). Schwann cell implantation reduced the size of cavitation and was sufficient to promote axonal regeneration (Kromer & Cornbrooks, 1985; Paino & Bunge, 1991). Furthermore, Schwann cells normally myelinate peripheral axons, improving fidelity and speed of signal transduction, and could potentially myelinate demyelinated CNS axons. Indeed, transplanted Schwann cells are able to myelinate axons (Blakemore & Franklin, 1991; Bunge, 1993; Tuszynski *et al.*, 1998). However, the effectiveness of

Schwann cells as a therapy is complicated by poor cell survival following implantation and observations that endogenous Schwann cells migrate in to the lesion site (Barakat *et al.*, 2005; Guest *et al.*, 2005; Pearse *et al.*, 2007).

The CNS counterpart of Schwann cells are oligodendrocytes. Oligodendrocytes fulfill a similar role in myelinating axons, are lost following injuring, and have also been shown to secrete neurotrophic factors (Wilkins *et al.*, 2001; Wilkins *et al.*, 2003). These neurotrophic factors complicate evaluations of the direct therapeutic benefits of oligodendrocyte transplantation as they are also sufficient to promote migration of endogenous Schwann cells (Blesch & Tuszynski, 2003; Cornejo *et al.*, 2010). Oligodendrocytes can be derived from neural precursor cells or embryonic stem cells cultured and differentiated to adopt an oligodendrocyte precursor cell (OPC) phenotype (Lu *et al.*, 2010; Sundberg *et al.*, 2010). Implantation of stem cell derived OPCs reported beneficial effects on spinal cord survival and behavior (Keirstead *et al.*, 2005; Sharp & Keirstead, 2009) but is not without criticism. First, the contusion model as an experimental paradigm potentially spares a number of tracts; this may explain the accelerated rate of recovery which cannot be attributed to regeneration. Second, control subjects received saline implantations which do not address the possibility that functional improvements are due to tissue preservation. A variety of cell types reduce lesion size and whether stem cell derived OPCs are more advantageous remains questionable. Further, logical contradictions question the therapeutic potentials of OPCs. Oligodendrocytes produce central myelin and central myelin is believed to be inhibitory

for regeneration and growth. Therefore, while oligodendrocyte implantations may promote remyelination, potential inhibitory effects on regeneration remain.

The pluriopotent nature of embryonic stem (ES) cell therapies have also garnered a significant amount of interest. While political and ethical issues continues to present barriers to scientific evaluation and advances, a limited number of human ES cells and induced pluripotent stem (iPS) cells have opened new avenues in this area of research. iPS cells and ES cells have been differentiated into neural restricted fates *in vitro* (Dimos *et al.*, 2008; Tsuji *et al.*, 2010). These neural precursors, in turn, are capable of differentiating into neuronal, oligodendritic, or astrocytic lineages. Potentially, neuronal differentiation and transplantation of neural precursors may generate relays to convey signals from injured axons to downstream targets. Alternatively, the introduction of neuronal restricted cells may by itself reconstitute the injured circuitry. However, these approaches have not yet produced promising results as survival of neural precursor cells is often poor and does not affect lesion cavity size (Vroemen *et al.*, 2003). Furthermore, transplanted neural precursors rarely adopt a neuronal fate, instead commonly differentiating in to astrocytes (Hofstetter *et al.*, 2005). Stem cell therapies remain a promising approach that require further investigation.

As a correlate of ES cells and iPS cells, embryonic spinal cord grafts have also been used in grafting studies. Embryonic spinal cords are considered immature, capable of robust growth, and sources of neurotrophic factors that support neuronal survival and growth. Initial experiments with embryonic spinal cords grafted tissue from E14 rats into a lesion cavity. Scientist observed axonal growth into and identified 5-HT

immunoreactive fibers in these transplants (Reier *et al.*, 1986). While there were claims of corticospinal regeneration into these transplants, the degree of growth and extent of penetration into the transplant was minimal (Schnell & Schwab, 1993). Greater success in promoting corticospinal regeneration has been achieved through the grafting of dissociated embryonic spinal cords (Lu unpublished) and presents avenues that require more investigation.

An additional cell type that garnered much attention in the course of studying remyelination and regeneration are olfactory ensheathing cells (OECs). OECs are glial cells associated with olfactory receptor neurons. Olfactory neurons have the unique characteristic of existing in a state of persistent replication and replacement. It is thought that OECs play a critical role in guiding these axons to their targets in the olfactory bulb. OECs have been associated with a number of roles including remyelination and regeneration. OECs in culture do not show an ability to remyelinate (Plant *et al.*, 2002). However, studies using alkaline phosphatase or GFP OECs demonstrate that transplanted OECs survive and myelinate axons following spinal cord injury (Franklin *et al.*, 1996; Akiyama *et al.*, 2004). OECs are also capable of secreting neurotrophic factors such as BDNF and NGF (Sasaki *et al.*, 2006) and may possess a neuroprotective effect. Indeed, the implantation of OECs into a CST lesion reduced the extent of neuronal atrophy in the M1 region of the cortex (Sasaki *et al.*, 2006). However, there is no apparent effect on axonal growth (Lu *et al.*, 2006).

Consideration in selecting a cell type for grafting are the availability of the cells, biological compatibility, and ease of acquisition. Utilizing bone marrow stromal cells

(BMSCs) or fibroblasts reduces such complexities associated with cell grafts. They are readily acquirable cells, can be expanded into large numbers, and are autologous, thereby negating the need for immunosuppression.

BMSCs have been used extensively as a cellular substrate and endogenously express trophic factors that reduce the degree of cystic cavitation and are permissive for regenerative (Hofstetter *et al.*, 2002; Novikova *et al.*, 2011; Vaquero & Zurita, 2011). In addition to innately expressed trophic factors, BMSCs are also amenable to genetic manipulation. BMSCs altered to express NT-3 significantly promote the regeneration of lesioned somatosensory neurons (Lu *et al.*, 2004). Furthermore, in conjunction with establishing a neurotrophin gradient beyond the lesion through viral transductions of cells, BMSCs are capable of promoting axonal bridging (Taylor *et al.*, 2006). Transgenic BMSCs have also been shown to promote sprouting of corticospinal axons caudal to a lesion (Tuszynski *et al.*, 2003).

Fibroblasts are another easily accessible source of autologous cells. Fibroblast can be isolated from patient skin puncture biopsies and rapidly proliferated. Genetically modified forms of fibroblast have an effect on behavioral outcomes (Suh *et al.*, 2011). Experiments utilizing fibroblast genetically modified to express NGF, BDNF, NT-3 or basic FGF found that sensory axons readily penetrated NGF, NT-3, and bFGF grafts while noradrenergic fibers were found only in NGF secreting grafts (Tuszynski *et al.*, 1994). The ease with which BMSCs and fibroblast may be acquired and robust effects they have demonstrated on axonal growth make them a more clinically relevant choice.

Extrinsic Factors Affecting Regeneration: Myelin Associated Inhibitors

Many axons are myelinated in order to facilitate rates of signal transduction and enhance signal fidelity. In the intact CNS, resident oligodendrocytes ensheath axons in myelin and are supportive for function. However, in the injured state, this very myelin becomes inhibitory to regaining function and is composed of a number of inhibitory molecules that prevent regeneration.

Nogo is among the most notable of myelin associated inhibitory molecules. There are three alternatively spliced forms of Nogo: Nogo-A, -B, and -C. All three isoforms share a common carboxy terminal and an inhibitory domain composed of 66 amino acid residues (Nogo-66) and are expressed on the myelin sheath of oligodendrocytes (Filbin, 2003). Nogo-A expresses a unique amino terminal that has been shown to have inhibitory properties and is expressed in oligodendrocytes but not by Schwann cells (Xie & Zheng, 2008). Interestingly, a means to neutralize Nogo was developed before Nogo itself was identified. Fractionation of myelin identified two fractions of 35 (NI-35) and 250 (NI-250) kD size that significantly inhibited neurite growth *in vitro* (GrandPre *et al.*, 2002). An antibody raised against these fractions, IN-1, showed growth promoting effects (Seijffers *et al.*, 2007). It was only later that that Nogo was identified as the primary component of myelin targeted by the IN-1 antibody and able to induce growth cone collapse (Chen *et al.*, 2000; GrandPre *et al.*, 2000; Prinjha *et al.*, 2000; Jankowski *et al.*, 2006; Chivatakarn *et al.*, 2007). IN-1 was found to specifically neutralize Nogo-A (Liebscher *et al.*, 2005). This inhibitory effect is accomplished through ligand binding to the Nogo receptor (NgR1) (Josephson *et al.*,

2002; Jankowski *et al.*, 2006). NgR1 exists as a membrane associated protein lacking a cytoplasmic domain. Consequently, signaling to intrinsic neuronal mechanisms and induction of growth cone collapse is dependent on the coreceptors TROY, LINGO, PirB, and p75 (Wang *et al.*, 2002a; Mi *et al.*, 2004; Park *et al.*, 2005; Shao *et al.*, 2005). Experiments aimed at neutralizing these inhibitory molecules *in vivo* applied IN-1 antibodies to injured spinal cords and reported increases in corticospinal regeneration and behavioral outcomes (Schnell & Schwab, 1993; Liebscher *et al.*, 2005). A function blocking peptide NEP1-40 was also generated to antagonize Nogo-66 inhibition. NEP1-40 administration demonstrated a significant degree of CST sprouting and rapid recovery of behavioral outcomes (GrandPre *et al.*, 2002; Li & Strittmatter, 2003). However, the rate of recovery suggests that regeneration does not account for the functional improvements and the trend of recovery in all groups suggest differences in lesion completeness. Furthermore, the ectopic labeling of axons in the spinal cord is suggestive of improper targeting of BDA tracer (Steward *et al.*, 2007).

To better understand the role of Nogo in axon regeneration, three groups independently and simultaneously generated *nogo* knockout mice. In one study of *nogo-A* deficient mice, CST regeneration into the scar tissue or beyond the lesion was not observed and Nogo-B was found to be upregulated through potential compensatory and redundant mechanism. A second group generated *nogo-A/B* and *nogo-A/B/C* knockout mice and observed a lack of regeneration following a CST lesion (Simonen *et al.*, 2003; Zheng *et al.*, 2005). The third group reported CST axon regeneratation in *nogo-A/B* deficient mice based on ectopic CST fibers and significant functional improvement (Kim

et al., 2003). However, these results have been questioned as the lesion model in the experiments was not sufficient to differentiate between spared and regenerating axons. Additionally, the findings were not reproducible by the other labs. Further, the observed ectopic axons were in fact the result of mis-targeted tracer injections that penetrated the ventricles (Steward *et al.*, 2007).

Myelin associated glycoprotein (MAG) is also a myelin associated molecule with inhibitory properties. MAG is present in PNS and CNS myelin and has a role in maintaining and compacting myelin. Surprisingly, MAG also signals through NgR1 and its associated coreceptors (Domeniconi *et al.*, 2002; Liu *et al.*, 2002). *In vitro* cultures with dorsal root ganglion and cerebellar neurons have demonstrated a significant inhibitory effect (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). Interestingly, MAG inhibition is dependent on the developmental state of the neuron. Embryonic neuron growth is promoted rather than inhibited by MAG (Turnley & Bartlett, 1998). The influence of MAG on inhibiting regeneration is complicated in light of *in vivo* studies using knockout mice. Only limited amounts of regeneration were observed following injury in these animals, suggesting that other inhibitory factors are present in the *in vivo* state (Bartsch *et al.*, 1995).

A third component of myelin with a history of inhibitory effects is oligodendrocyte myelin glycoprotein (Omgp). Like MAG, Omgp is found in both the CNS and PNS and is expressed by both oligodendrocytes and neurons. Similarly, Omgp was also found to signal through NgR1 (Wang *et al.*, 2002b). Experiments to localize Omgp found that it was largely expressed at the Nodes of Ranvier (Huang *et al.*, 2005).

Studies have demonstrated that Omgp is also able to promote the collapse of growth cones and inhibit growth (Kottis *et al.*, 2002).

The promiscuity of the Nogo receptor allows for greater interpretations of the lack of regeneration in knockout mice studies and casts doubt on early experiments claiming regeneration. Perhaps the reason for the lack of regeneration was due to redundancy in ligands for the receptor. Therefore, it possible that elimination of components for this common receptor may yield greater results than elimination of a single ligand. The generation of a NgR knockout mice would, in theory, prevent inhibition from Nogo, MAG, and Omgp. In vitro studies using such mice has demonstrated an expected effect on growth cone collapse (Kim *et al.*, 2004; Chivatakarn *et al.*, 2007). However, NgR1 knockout did not affect growth on a myelin substrate. *In vivo* studies using these mice observed enhancements in regeneration of rubrospinal and raphespinal axons in a full transection model (Kim *et al.*, 2004). However, labeled rubrospinal axons were ectopically found in specific ventral regions of the spinal cord corresponding to the location of often spared ventral CST fibers. Furthermore, animals showed an improvement in behavior 2 days following lesion. These observations suggest that the transection may have been incomplete and difference in lesion completeness complicate the interpretation of the results. A separate study that also genetically deleted NgR demonstrated NgR does not inhibit axonal growth or block CST regeneration *in vivo*. Furthermore, it was suggested that homologues of NgR1, such as NgR2 and NgR3, may be upregulated following downregulation of NgR1 (Zheng *et al.*, 2005). NgR2 has been shown to respond to MAG and promote growth cone collapse (Venkatesh *et al.*, 2005).

An alternative means to block NgR signaling is through the downregulation or knockdown of p75. p75 is a low affinity neurotrophin tyrosine receptor kinase that has the potential to bind BDNF, NT-3, or NGF. It was also found to coprecipitate with MAG (Yamashita *et al.*, 2002). Further investigation revealed that it is one the cytoplasmic components that enable signaling through NgR (Wang *et al.*, 2002a; Wong *et al.*, 2002). In p75 neutralization experiments, there is *in vitro* release from myelin induced growth cone collapse (Wang *et al.*, 2002a). Unfortunately, *in vivo* studies demonstrated that p75NTR knockouts did not possess greater regenerative potential of CST or ascending sensory fibers (Song *et al.*, 2004; Zheng *et al.*, 2005). A potential explanation for the lack of regeneration is the existence of additional coreceptors that propagate the NgR signal. These coreceptors include the proteins LINGO and TROY. *In vivo* experiments using LINGO-FC as a dominant negative (Ji *et al.*, 2006) suggest it promotes regeneration of RST and CST axons but concerns remain regarding the completeness of lesion based on sparing of tissue and subjective nonlinear assessments of behavior using the Basso, Beattie, and Bresnahan (BBB) scale in a range that is difficult to discern.

The lack of regeneration observed in subjects deficient in NgR, p75NTR, or LINGO may be explained by the existence of an additional receptor capable of transducing the Nogo signal. This rationale may also explain why studies using a triple knockout for MAG, Nogo, and Omgp, the known ligands of NgR, failed to elicit regeneration (Lee *et al.*, 2010). This receptor was identified through expression cloning to be the paired immunoglobulin-like receptor B (PirB) and was also sensitive to Nogo, MAG, and Omgp (Atwal *et al.*, 2008). *In vitro* blocking of PirB partially rescued neurite

inhibition; combined PirB blocking and Nogo neutralization resulted in almost complete rescue. *In vivo* studies with PirB knockouts failed to observe regenerative or functional improvements (Nakamura *et al.*, 2011). Once again, this may be the result of redundancy in receptors for Nogo, MAG, and Omgp.

Extrinsic Factors Affecting Regeneration: Guidance Molecules

During development, axonal growth must be guided correctly to their appropriate targets. This is achieved by secreted or membrance associated factors that generate gradients to which growth cones respond. Following maturation, many of these guidance molecules are downregulated. However, some neurons continue to express receptors for these guidance molecules long after development, suggesting a possible role for these same molecules in the adult nervous system (Giger *et al.*, 2010). Commonly, these axon guidance molecules are also associated with myelin.

Semaphorins are a class of guidance molecules that play a pivotal role during development and also have a role in the mature nervous system (Reza *et al.*, 1999). Developmentally, semaphorins function as repulsive cues and exist as eight classes of membrane associated or secreted proteins that signal through neuropilin or plexins (Fiore & Puschel, 2003). Class 3 semaphorins (Sema3) are expressed following injury and retain their repulsive effects on axon growth cones (Reza *et al.*, 1999). The source of secreted Sema3 in injury models is believed to be a component of the glial scar (Pasterkamp *et al.*, 1999; Pasterkamp *et al.*, 2001). Application of Sema3 in an *in vivo* model demonstrated

an inhibitory effect on small diameter DRG sprouting and mechanical allodynia (Tang *et al.*, 2004; Hayashi *et al.*, 2011). Interestingly, large-diameter NT-3 sensitive DRGs like those of the somatosensory system are not affected by Sema3 *in vitro* (Pasterkamp *et al.*, 2001). An inhibitor of Sema3, SMI-216289, was found to block Sema3 induced repulsion of DRGs in vitro and accelerate regeneration of axotomized olfactory nerves (Kaneko *et al.*, 2006). In these same experiments, inhibition of Sema3A was reported to enhance regeneration. However, there is controversy in terms of lesion demarcation and behavioral data that shows functional improvement within 3 days, a period too short for anatomical change to occur. A number of the other semaphorins have been identified to influence growth cone collapse and to be expressed in the *in vivo* state. Sema4D is expressed by oligodendrocytes following spinal cord injury and has inhibitory effects on postnatal sensory and cerebellar granule neurons (Moreau-Fauvarque *et al.*, 2003).Sema5A has been shown to induce growth cone collapse and is also expressed by oligodendrocytes in the optic nerve (Goldberg *et al.*, 2004).

Netrins are another class of guidance molecules that have been shown to have effects on adult neuronal growth. Netrins are secreted proteins that affect growing axons by attracting or repelling their direction. Attraction is mediated by the UNC-40/DCC receptor while repulsion is mediated by the UNC5 receptor (Moore *et al.*, 2007). Netrin-1 is expressed in the CNS, especially at the nodes of Ranvier, and corticospinal and rubrospinal projection express the repulsive receptor UNC5. Neutralizing netrin-1 signaling with UNC5 receptor bodies promotes spinal motor neuron growth on myelin *in*

vitro. Implantation of fibroblast grafts expressing netrin-1 inhibits the growth of UNC5 expressing fibers (Low *et al.*, 2008).

Ephrins are also developmental guidance molecules that are persistently expressed in the adult nervous system (Liebl *et al.*, 2003). Transduction of of ephrin signaling is conveyed through binding to EphA and EphB receptors. Eph receptor signaling often has repulsive results in development and is also preserved in the adult state. Following spinal cord injury, a number of Eph receptors including EphA3, A4, and A7 are transcriptionally upregulated (Willson *et al.*, 2002). Myelinating oligodendrocytes express ephrin-B3 in the adult spinal cord and it is an inhibitor of cortical neurite growth. This effect is dependent on EphA4 (Benson *et al.*, 2005). EphA4 concentrates in the distal process of lesioned corticospinal axons and ephrin-B2, another ligand for EphA4, is upregulated in astrocytes (Fabes *et al.*, 2006). The same group blocked EphA4 signaling with a peptide and found that the peptide enhanced CST sprouting but was insufficient to promote regeneration across the lesion site (Fabes *et al.*, 2007). Correspondingly, reducing EphA4 expression with antisense oligonucleotides did not result in significant improvements in CST fiber regeneration or physiological responses by transcranial magnetic motor evoked potentials (Cruz-Orengo *et al.*, 2007). In contrast, EphB3 and ephrinB3 were found to be attractive promoters of axon growth. In retinal ganglion cells following injury, RGCs expressed ephrinB3 and macrophages expressed EphB3. *In vitro*, EphB3 supported adult RGC outgrowth while *in vivo*, loss of EphB3 reduced axonal plasticity (Liu *et al.*, 2006).

Extrinsic Factors Affecting Regeneration: Loss of Neurotrophic Support

Neurotrophins are classically secreted molecules that influence neuronal development and axon growth. The classical neurotrophic factors are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Neurotrophins are translated as proneurotrophins which lack the efficacy of the mature form. Neurotrophin maturation occurs through proteolytic cleavage and homodimerization to produce 13 kD proteins with potent cellular effects (Lee *et al.*, 2001). Neurotrophin effects are transduced via binding to tropomysoin-receptor kinases (trk) and the dimerization of trk receptors. The neurotrophin receptors exhibit specific ligand affinities: trkA binds NGF, trkB binds BDNF and NT-4/5, and trkC binds NT-3(Bibel & Barde, 2000). Additionally, there exists a non-selective receptor with the potential to bind all four neurotrophins with lower affinity, the p75 receptor. Expression of p75 with another trk modulates trk signaling activity and generates a high-affinity receptor state in neurons that express both p75 and trk. In the condition of isolated p75 expression, neurotrophin signaling produces cell death via apoptosis (Huang & Reichardt, 2001).

The source of neurotrophin production is the target with which it creates a synaptic connection. When axons are lesioned, the loss of this postsynaptic source of neurotrophic factors results in axon retraction and cell death (Tobias *et al.*, 2003). Further evidence for importance of neurotrophins can be found in the PNS which is able to successfully regenerate. Following injury to a peripheral nerve, Schwann cells distal to a lesion express a variety of neurotrophic factors that are able to sustain neuronal survival

and axonal growth (Meyer *et al.*, 1992; Funakoshi *et al.*, 1993). In the CNS, an upregulation of neurotrophic factors expression is not observed following injury (Plunet *et al.*, 2002). These findings suggested that application of neurotrophic factors to a CNS lesion may similarly promote cell survival and axon growth.

Early studies demonstrated a requirement for neurotrophin for proper neuronal development and survival (Ernfors *et al.*, 1994a; Ernfors *et al.*, 1994b). In compartmentalized culture studies, the removal of neurotrophins from the axonal compartment resulted in axon retraction and degeneration (Campenot, 1977). Following maturation, target acquisition, and synapse formation, neurons downregulate trk receptor expression and, thereby, have reduced responses to neurotrophin (Molliver *et al.*, 1997). However, neurotrophins are still influential in the adult state. In an adult model in which the cholinergic neurons of the basal forebrain were lesioned, the application of NGF was able to prevent cell death (Kromer, 1987). Furthermore, the application of BDNF is sufficient to prevent apoptosis of lesioned corticospinal motor neurons in a subcortical lesion model that usually results in at least 50% cell loss (Giehl & Tetzlaff, 1996) and in a spinal cord injury model (Brock *et al.*, 2010). The rubrospinal tract (RST) similarly responsd to BDNF and RST neuron survival following a lesion can be augmented with BDNF application (Kobayashi *et al.*, 1997).

In addition to effects on survival, neurotrophins have also demonstrated the ability to affect axonal growth. Following lesion of spinal cord, NGF is able to promote the growth of a number of neuronal populations including local cholinergic motor neurons, ascending sensory neurons, and tyrosine-hydroxylase (TH) positive coerulospinal

neurons (Tuszynski *et al.*, 1996). Grafting of BDNF secreting cells is sufficient to promote growth of serotonergic, coerulospinal, and large-diameter ascending sensory neurons (Lu *et al.*, 2005). Furthermore, overexpression of trkB and grafting of BDNF secreting cells in a subcortical lesion model promotes the regeneration of lesioned CST neurons (Hollis *et al.*, 2009). NT-3 expression promotes ascending sensory axons regeneration and sprouting of spared CST into gray matter (Grill *et al.*, 1997; Taylor *et al.*, 2006). The extent with which neurotrophic factor promote axon growth emphasizes the importance of neurotrophins for achieving spinal cord regeneration. While the application of a single factor alone is not sufficient to produce robust regeneration, perhaps a combinatorial approach that includes neurotrophic factors will result in consistent and robust functional regeneration.

Intrinsic Factors Affecting Regeneration

Exhaustive studies of extrinsic factors have provided valuable information regarding the potential of extrinsic manipulations to elicit regeneration. While modulation of extrinsic factors have met with some success, it is clear that environmental manipulations alone will be insufficient to promote the extensive degrees of regeneration required for significant functional improvement. Intrinsic factors may provide what is needed to achieve this regeneration. Once again, though there are interactions between intrinsic and extrinsic factors, this historical division has guided a number of studies to identify important components of axon growth. The bases for these studies have involved observations of a biological response known as the peripheral conditioning lesion and the

capacity of immature neurons for growth. The rationale and current status of these approaches will be discussed in the following sections.

Intrinsic Factors Affecting Regeneration: Expression of Regeneration Associated Genes

The peripheral conditioning effect allows for impressive degrees of regeneration and is observed only in dorsal root ganglion (DRG) neurons, a neuron that exists in a unique environment between the CNS and PNS and is responsible for conveying sensory information from targeted organs to the CNS. The DRG soma is located within the PNS and extends a process peripherally and another branch centrally. Following lesion of the peripheral branch, there is significant regeneration and reinnervation of lost peripheral targets. Following lesion of the central branch, similar levels of regenerationare not observed. However, if the peripheral branch is lesioned prior to a central lesion, the neuron becomes "conditioned" and subsequent injury to the central process will be met with enhanced degrees of regeneration (Richardson & Issa, 1984; Neumann & Woolf, 1999). Furthermore, conditioned neurons are no longer inhibited by myelin. Because the peripheral lesion does not affect the state of environment within the CNS, it is believed that the neuron undergoes intrinsic changes and these specific changes are of particular interest to the regeneration field.

The early changes that occur following conditioning are dependent on transcription. Inhibition of transcription abolishes improvements in growth observed with

a conditioning (Smith & Skene, 1997; Qiu *et al.*, 2002). Studies to identify the specific genetic changes found that expression levels change in hundreds of genes (Costigan *et al.*, 2002). Some of these changes are related to general neuronal injury signals or to processes associated with growth but by themselves are not sufficient to promote growth. Growth associated protein-43 (GAP-43) is highly upregulated in peripherally conditioned neurons but not in centrally injured neurons (Schreyer & Skene, 1993). However, overexpression of GAP-43 is insufficient to promote growth on a myelin substrate. However, studies have identified key components of the conditioning lesion that are necessary for successful regeneration. Levels of cyclic adenosine monophosphates (cAMP) increase within 24 hours following a peripheral conditioning lesion (Qiu *et al.*, 2002). Furthermore, there is increased activity by protein kinase A (PKA), a cAMP-dependent protein kinase, following peripheral injury. The effects observed by the peripheral conditioning are dependent on this initial upregulation of PKA. Elevating cAMP levels with a nonhydrolizable form, dibutyryl-cAMP (db-cAMP), enhances regeneration but is not as robust as the conditioning effect (Neumann *et al.*, 2002). Additionally, similar to conditioned neurons, db-cAMP treated neurons are released from myelin inhibition (Neumann *et al.*, 2002; Qiu *et al.*, 2002).

The genetic changes resulting from the conditioning effect are slowly being identified. cAMP elevation is capable of driving expression of a number of genes. One such factor is the enzyme arginase-1 which is required in the production of spermidine (Cai *et al.*, 2002). Like treatment with cAMP, elevation of spermidine levels were sufficient to promote growth and overcome myelin inhibition (Deng *et al.*, 2009).

SMAD1 is necessary for the conditioning effect and that activation of SMAD1 via BMP2 and BMP4 treatment can recapitulate the conditioning effect (Zou *et al.*, 2009). Transgenic mice that constitutively expressed activating transcription factor 3 (ATF3) increased DRG neurite elongation on permissive substrates but did not overcome myelin inhibition or promote *in vivo* regeneration (Seijffers *et al.*, 2007). Sox11 is a transcription factor expressed by developing and injured DRGs and is required for cell survival and neurite growth (Jankowski *et al.*, 2006).

While specific components that contribute to the conditioning effect have been identified, additional work is needed to isolate the key regulators.

Intrinsic Factors Affecting Regeneration: Developmental Mechanisms

Embryonic neural tissue possesses an innate ability for growth that is not seen in adult neural tissue. Studies demonstrate that embryonic neural tissue transplanted into the adult cortex survives and integrates with cortical circuitry (Gonzalez *et al.*, 1988; Sorensen *et al.*, 1996). In these early experiment, host-graft connectivity was demonstrated by tracer injections; however, tracer are not specific for differentiating grafted cells from host fibers (Gonzalez *et al.*, 1988; Sorensen *et al.*, 1996). Taking advantage of GFP transgenic mice, replication of these experiments suggest that grafts of embryonic cortex into the adult CNS integrate with the host CNS and project axons considerable distance down the spinal cord (Gaillard *et al.*, 2007). However, interpretations of these results are complicated by potential cell fusion that may impart

fluorescence to host corticospinal fibers, giving the false impression of integration and growth from transgenic grafts. While the authors attempted to address cell fusion, the methods used may lack sensitivity in detecting such an event (Tuszynski, 2007).

Complementing embryonic cortical grafts, embryonic spinal cord grafts have also been attempted. In these studies, host fibers were observed penetrating embryonic tissue grafts; however, this penetration was limited (Jakeman & Reier, 1991; Diener & Bregman, 1998). Studies using GFP transgenic animals have replicated these early grafting experiments to better visualize host and graft cells. In this case, transplanted embryonic cells exhibited extensive growth into host tissue. Furthermore, corticospinal fibers were observed penetrating the embryonic graft (Lu unpublished).

Growth of embryonic grafts in the inhibitory adult environment suggests that the genetic state of these neurons differ from their adult counterparts and studies of these developmentally regulated growth associated genes have identified promising candidates. One candidate under intense study is phosphatase and tensin homolog (PTEN). PTEN is a negative regulator of the mammalian target of rapamycin (mTOR) pathway, a pathway that controls cell growth and size through modulation of protein translation (Liu *et al.*, 2010b). Following maturation, mTOR is downregulated by expression of PTEN. In conditional knockouts of PTEN in retinal ganglion cells, robust regeneration was observed following optic nerve injury (Park *et al.*, 2008). In models of severe peripheral nerve transection, pharmacological inhibition and siRNA induced knockdown of PTEN accelerates axon growth (Christie *et al.*, 2010). However, PTEN knockdown is most notable for effects on CST growth. In these studies, investigators conditionally deleted

PTEN and lesioned the CST with either a pyramidotomy, dorsal hemisection, or complete crush injury. They found that PTEN deletion enhanced sprouting from spared CST axons and promoted regeneration of lesioned CST fibers beyond the lesion in both hemisection and complete crush injuries (Liu *et al.*, 2010a).

Though these results are promising, they must be met with critical optimism. First, many of the regenerated axons were associated with GFAP immunoreactive tissue bridges. While it is completely possible that PTEN deletion promotes CST regeneration, the possibility that enhanced sprouting from spared axons within GFAP bridges cannot be discounted. Second, PTEN was deleted in mice at 4 weeks of age. While this is considerably different from embryonic and early postnatal animals, animals this age have not reached adulthood and CST axons have the potential to undergo considerable plastic changes. Therefore, PTEN deletion in this state may simply enhance an already elevated intrinsic state of growth. Third, quantification of CST axons depends on the actively transported tracer BDA. If PTEN deletion enhances active transport mechanisms, a greater abundance of BDA within axons would increase sensitivity in detecting fine caliber fibers that are normally missed in control animals and may potentially account for the increased numbers of fibers observed. Should these results be replicated in older animals using a complete lesion model and an axonal tracer that is not dependent on active transport mechanisms, PTEN deletion would be a very promising candidate to promote CST regeneration following spinal cord injury.

Combinatorial Approaches

The exhausitive exploration of extrinsic factors and studies of intrinsic factors for promoting growth have revealed the complexity involved in inducing axon regeneration. While single factor manipulations are informative with regards to providing information on sufficiency and a basic understanding of mechanism, the behavioral effects of single factor manipulations are limited. As such, it is understood that in order to achieve significant regeneration, combinatorial approaches to neutralize inhibitory factors and upregulate growth associated mechanisms are necessary. To an extent, single manipulations may influence a number of factors that influence regeneration. Grafting of cells replaces lost cellular substrate but may also provide trophic support in the form of secreted molecules. However, a true combinatorial approach should attempt to address as many of the aforementioned factors as possible to achieve significant functional improvement following SCI.

There are examples of such approaches demonstrating that combinatorial treatments are more effective than individual manipulations. While cAMP elevation mimics properties of the conditioning lesion, a greater degree of ascending sensory neuron regeneration and bridging is achievable when cAMP elevation is combined with MSC grafts to provide a physical bridge across a lesion cavity and neurotrophic support rostral to the lesion (Lu *et al.*, 2004). Combinations of peripheral nerve grafts providing a cellular bridge and neurotrophic support and digestion of inhibitory CSPGs at the caudal end of integration increases the number of supraspinal fibers that reenter the CNS and enhances behavioral outcome when compared to peripheral nerve grafts alone (Houle *et al.*, 2006b).

The results observed with these combinatorial treatments exceed what is observed with isolated treatments and speaks to the effectiveness of combinatorial approaches. However, the outcomes are still far from what is desired. Hence, further studies to identify extrinsic and intrinsic factors are necessary in order to provide the foundation and tools from which to achieve effective combinatorial therapies for spinal cord regeneration.

Rationale and hypotheses

As neurons mature, their genetic expressions pattern change and these changes correlate with a reduction in capacity for growth. This is in contrast to developing neurons which express a unique genetic profile that enables enhanced axonal growth and growth in inhibitory environments. The focus of this work is to identify developmentally regulated mechanisms that promote axon growth and assess their potential to promote growth in adult neurons and as therapeutic interventions in an *in vivo* model of spinal cord injury.

Studies of axon development suggest that TGF-β is necessary for axon differentiation. Genetic mouse models eliminating the type II TGF-β receptor demonstrate that developing cortical neurons lacking TGF-β signaling fail to migrate properly to the cortical plate and, in spite of a dynamic leading-edge, fail to exhibit axonal growth at the trailing-edge. The same group also demonstrated that TGF-β receptors are polarized to the axon during development and that TGF-β signaling is

sufficient to specify axon differentiation (Yi *et al.*, 2010). The developmentally regulated transcriptional regulator ski-like novel protein (SnoN) is a component of the TGF-β signaling pathway and promotes elongation specifically in the axon of postnatal cerebellar granule neurons (Stegmuller *et al.*, 2006). The aim of this work is determine whether developmentally important TGF-β signaling pathways are conserved in adult neurons and whether modulation of this signaling cascade by SnoN over-expression is sufficient to promote regeneration following spinal cord injury.

Chapter 2 investigates the hypothesis that modulation of TGF-β signaling, a pathway critical for axonal differentiation during development, will enhance regeneration of adult neurons following spinal cord injury.

References

Akiyama, Y., Lankford, K., Radtke, C., Greer, C.A. & Kocsis, J.D. (2004) Remyelination of spinal cord axons by olfactory ensheathing cells and Schwann cells derived from a transgenic rat expressing alkaline phosphatase marker gene. *Neuron Glia Biol*, **1**, 47-55.

Antony, J.M., van Marle, G., Opii, W., Butterfield, D.A., Mallet, F., Yong, V.W., Wallace, J.L., Deacon, R.M., Warren, K. & Power, C. (2004) Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nature neuroscience*, **7**, 1088-1095.

Atwal, J.K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C. & Tessier-Lavigne, M. (2008) PirB is a functional receptor for myelin inhibitors of axonal regeneration. *Science*, **322**, 967-970.

Baichwal, R.R., Bigbee, J.W. & DeVries, G.H. (1988) Macrophage-mediated myelin-related mitogenic factor for cultured Schwann cells. *Proc Natl Acad Sci U S A*, **85**, 1701-1705.

Banati, R.B., Gehrmann, J., Schubert, P. & Kreutzberg, G.W. (1993) Cytotoxicity of microglia. *Glia*, **7**, 111-118.

Bandtlow, C.E. & Zimmermann, D.R. (2000) Proteoglycans in the developing brain: new conceptual insights for old proteins. *Physiol Rev*, **80**, 1267-1290.

Baptiste, D.C., Powell, K.J., Jollimore, C.A., Hamilton, C., LeVatte, T.L., Archibald, M.L., Chauhan, B.C., Robertson, G.S. & Kelly, M.E. (2005) Effects of minocycline and tetracycline on retinal ganglion cell survival after axotomy. *Neuroscience*, **134**, 575-582.

Barakat, D.J., Gaglani, S.M., Neravetla, S.R., Sanchez, A.R., Andrade, C.M., Pressman, Y., Puzis, R., Garg, M.S., Bunge, M.B. & Pearse, D.D. (2005) Survival, integration, and axon growth support of glia transplanted into the chronically contused spinal cord. *Cell Transplant*, **14**, 225-240.

Bartsch, U., Bandtlow, C.E., Schnell, L., Bartsch, S., Spillmann, A.A., Rubin, B.P., Hillenbrand, R., Montag, D., Schwab, M.E. & Schachner, M. (1995) Lack of

evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. *Neuron*, **15**, 1375-1381.

Benson, M.D., Romero, M.I., Lush, M.E., Lu, Q.R., Henkemeyer, M. & Parada, L.F. (2005) Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 10694-10699.

Beuche, W. & Friede, R.L. (1984) The role of non-resident cells in Wallerian degeneration. *J Neurocytol*, **13**, 767-796.

Bibel, M. & Barde, Y.A. (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes & development*, **14**, 2919-2937.

Blakemore, W.F. & Franklin, R.J. (1991) Transplantation of glial cells into the CNS. *Trends Neurosci*, **14**, 323-327.

Blesch, A. & Tuszynski, M.H. (2003) Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination. *The Journal of comparative neurology*, **467**, 403-417.

Bovolenta, P. & Fernaud-Espinosa, I. (2000) Nervous system proteoglycans as modulators of neurite outgrowth. *Prog Neurobiol*, **61**, 113-132.

Bracken, M.B. (1991) Treatment of acute spinal cord injury with methylprednisolone: results of a multicenter, randomized clinical trial. *J Neurotrauma*, **8 Suppl 1**, S47-50; discussion S51-42.

Bracken, M.B., Shepard, M.J., Collins, W.F., Holford, T.R., Young, W., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J. & et al. (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med*, **322**, 1405-1411.

Bracken, M.B., Shepard, M.J., Collins, W.F., Jr., Holford, T.R., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J.C., Marshall, L.F. & et al. (1992) Methylprednisolone or naloxone treatment after acute spinal cord injury: 1-year

follow-up data. Results of the second National Acute Spinal Cord Injury Study. *J Neurosurg*, **76**, 23-31.

Bracken, M.B., Shepard, M.J., Holford, T.R., Leo-Summers, L., Aldrich, E.F., Fazl, M., Fehlings, M., Herr, D.L., Hitchon, P.W., Marshall, L.F., Nockels, R.P., Pascale, V., Perot, P.L., Jr., Piepmeier, J., Sonntag, V.K., Wagner, F., Wilberger, J.E., Winn, H.R. & Young, W. (1997) Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA*, **277**, 1597-1604.

Bradbury, E.J., Moon, L.D., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W. & McMahon, S.B. (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature*, **416**, 636-640.

Brock, J.H., Rosenzweig, E.S., Blesch, A., Moseanko, R., Havton, L.A., Edgerton, V.R. & Tuszynski, M.H. (2010) Local and remote growth factor effects after primate spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 9728-9737.

Bunge, R.P. (1993) Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. *Curr Opin Neurobiol*, **3**, 805-809.

Bunge, R.P. (1994) The role of the Schwann cell in trophic support and regeneration. *J Neurol*, **242**, S19-21.

Bycroft, J., Shergill, I.S., Chung, E.A., Arya, N. & Shah, P.J. (2005) Autonomic dysreflexia: a medical emergency. *Postgrad Med J*, **81**, 232-235.

Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R.R. & Filbin, M.T. (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. *Neuron*, **35**, 711-719.

Campenot, R.B. (1977) Local control of neurite development by nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, **74**, 4516-4519.

Carlson, G.D., Gorden, C.D., Oliff, H.S., Pillai, J.J. & LaManna, J.C. (2003) Sustained spinal cord compression: part I: time-dependent effect on long-term pathophysiology. *J Bone Joint Surg Am*, **85-A**, 86-94.

Chan, P.H. (2004) Mitochondria and neuronal death/survival signaling pathways in cerebral ischemia. *Neurochem Res*, **29**, 1943-1949.

Chen, D., Apple, D.F., Jr., Hudson, L.M. & Bode, R. (1999) Medical complications during acute rehabilitation following spinal cord injury--current experience of the Model Systems. *Arch Phys Med Rehabil*, **80**, 1397-1401.

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

Chivatakarn, O., Kaneko, S., He, Z., Tessier-Lavigne, M. & Giger, R.J. (2007) The Nogo-66 receptor NgR1 is required only for the acute growth cone-collapsing but not the chronic growth-inhibitory actions of myelin inhibitors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 7117-7124.

Christie, K.J., Webber, C.A., Martinez, J.A., Singh, B. & Zochodne, D.W. (2010) PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 9306-9315.

Claxton, A.R., Wong, D.T., Chung, F. & Fehlings, M.G. (1998) Predictors of hospital mortality and mechanical ventilation in patients with cervical spinal cord injury. *Can J Anaesth*, **45**, 144-149.

Cornejo, M., Nambi, D., Walheim, C., Somerville, M., Walker, J., Kim, L., Ollison, L., Diamante, G., Vyawahare, S. & de Bellard, M.E. (2010) Effect of NRG1, GDNF, EGF and NGF in the migration of a Schwann cell precursor line. *Neurochemical research*, **35**, 1643-1651.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E. & Woolf, C.J. (2002) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated

genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci*, **3**, 16.

Cruz-Orengo, L., Figueroa, J.D., Torrado, A., Puig, A., Whittemore, S.R. & Miranda, J.D. (2007) Reduction of EphA4 receptor expression after spinal cord injury does not induce axonal regeneration or return of tcMMEP response. *Neuroscience letters*, **418**, 49-54.

Dalyan, M., Sherman, A. & Cardenas, D.D. (1998) Factors associated with contractures in acute spinal cord injury. *Spinal Cord*, **36**, 405-408.

Daverat, P., Gagnon, M., Dartigues, J.F., Mazaux, J.M. & Barat, M. (1989) Initial factors predicting survival in patients with a spinal cord injury. *J Neurol Neurosurg Psychiatry*, **52**, 403-406.

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

Deng, K., He, H., Qiu, J., Lorber, B., Bryson, J.B. & Filbin, M.T. (2009) Increased synthesis of spermidine as a result of upregulation of arginase I promotes axonal regeneration in culture and in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **29**, 9545-9552.

DeVivo, M.J., Black, K.J., Richards, J.S. & Stover, S.L. (1991) Suicide following spinal cord injury. *Paraplegia*, **29**, 620-627.

Diener, P.S. & Bregman, B.S. (1998) Fetal spinal cord transplants support growth of supraspinal and segmental projections after cervical spinal cord hemisection in the neonatal rat. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **18**, 779-793.

Dimar, J.R., 2nd, Glassman, S.D., Raque, G.H., Zhang, Y.P. & Shields, C.B. (1999) The influence of spinal canal narrowing and timing of decompression on neurologic recovery after spinal cord contusion in a rat model. *Spine (Phila Pa 1976)*, **24**, 1623-1633.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., Wichterle, H., Henderson, C.E. &

Eggan, K. (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science*, **321**, 1218-1221.

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

Elkabes, S., DiCicco-Bloom, E.M. & Black, I.B. (1996) Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J Neurosci*, **16**, 2508-2521.

Ernfors, P., Lee, K.F. & Jaenisch, R. (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature*, **368**, 147-150.

Ernfors, P., Lee, K.F., Kucera, J. & Jaenisch, R. (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell*, **77**, 503-512.

Fabes, J., Anderson, P., Brennan, C. & Bolsover, S. (2007) Regeneration-enhancing effects of EphA4 blocking peptide following corticospinal tract injury in adult rat spinal cord. *The European journal of neuroscience*, **26**, 2496-2505.

Fabes, J., Anderson, P., Yanez-Munoz, R.J., Thrasher, A., Brennan, C. & Bolsover, S. (2006) Accumulation of the inhibitory receptor EphA4 may prevent regeneration of corticospinal tract axons following lesion. *The European journal of neuroscience*, **23**, 1721-1730.

Faulkner, J.R., Herrmann, J.E., Woo, M.J., Tansey, K.E., Doan, N.B. & Sofroniew, M.V. (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*, **24**, 2143-2155.

Fawcett, J.W., Curt, A., Steeves, J.D., Coleman, W.P., Tuszynski, M.H., Lammertse, D., Bartlett, P.F., Blight, A.R., Dietz, V., Ditunno, J., Dobkin, B.H., Havton, L.A., Ellaway, P.H., Fehlings, M.G., Privat, A., Grossman, R., Guest, J.D., Kleitman, N., Nakamura, M., Gaviria, M. & Short, D. (2007) Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: spontaneous recovery after spinal cord injury and statistical power needed for therapeutic clinical trials. *Spinal Cord*, **45**, 190-205.

Fehlings, M.G. & Baptiste, D.C. (2005) Current status of clinical trials for acute spinal cord injury. *Injury*, **36 Suppl 2**, B113-122.

Fehlings, M.G. & Perrin, R.G. (2005) The role and timing of early decompression for cervical spinal cord injury: update with a review of recent clinical evidence. *Injury*, **36 Suppl 2**, B13-26.

Filbin, M.T. (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci*, **4**, 703-713.

Fiore, R. & Puschel, A.W. (2003) The function of semaphorins during nervous system development. *Front Biosci*, **8**, s484-499.

Fitch, M.T., Doller, C., Combs, C.K., Landreth, G.E. & Silver, J. (1999) Cellular and molecular mechanisms of glial scarring and progressive cavitation: in vivo and in vitro analysis of inflammation-induced secondary injury after CNS trauma. *J Neurosci*, **19**, 8182-8198.

Franklin, R.J., Gilson, J.M., Franceschini, I.A. & Barnett, S.C. (1996) Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult CNS. *Glia*, **17**, 217-224.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V.M. & Persson, H. (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J Cell Biol*, **123**, 455-465.

Furlan, J.C. & Fehlings, M.G. (2008) Cardiovascular complications after acute spinal cord injury: pathophysiology, diagnosis, and management. *Neurosurg Focus*, **25**, E13.

Gaillard, A., Prestoz, L., Dumartin, B., Cantereau, A., Morel, F., Roger, M. & Jaber, M. (2007) Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons. *Nature neuroscience*, **10**, 1294-1299.

Garcia-Alias, G., Barkhuysen, S., Buckle, M. & Fawcett, J.W. (2009) Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. *Nature neuroscience*, **12**, 1145-1151.

Giehl, K.M. & Tetzlaff, W. (1996) BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. *The European journal of neuroscience*, **8**, 1167-1175.

Giger, R.J., Hollis, E.R., 2nd & Tuszynski, M.H. (2010) Guidance molecules in axon regeneration. *Cold Spring Harb Perspect Biol*, **2**, a001867.

Giulian, D. (1993) Reactive glia as rivals in regulating neuronal survival. *Glia*, **7**, 102-110.

Glickman, S. & Kamm, M.A. (1996) Bowel dysfunction in spinal-cord-injury patients. *Lancet*, **347**, 1651-1653.

Goldberg, J.L., Vargas, M.E., Wang, J.T., Mandemakers, W., Oster, S.F., Sretavan, D.W. & Barres, B.A. (2004) An oligodendrocyte lineage-specific semaphorin, Sema5A, inhibits axon growth by retinal ganglion cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 4989-4999.

Gonzalez, M.F., Sharp, F.R. & Loken, J.E. (1988) Fetal frontal cortex transplanted to injured motor/sensory cortex of adult rats: reciprocal connections with host thalamus demonstrated with WGA-HRP. *Experimental neurology*, **99**, 154-165.

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

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

Grill, R., Murai, K., Blesch, A., Gage, F.H. & Tuszynski, M.H. (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **17**, 5560-5572.

Grimpe, B. & Silver, J. (2002) The extracellular matrix in axon regeneration. *Prog Brain Res*, **137**, 333-349.

Guest, J.D., Hiester, E.D. & Bunge, R.P. (2005) Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury. *Experimental neurology*, **192**, 384-393.

Hagg, T. & Oudega, M. (2006) Degenerative and spontaneous regenerative processes after spinal cord injury. *J Neurotrauma*, **23**, 264-280.

Harvey, L.A. & Herbert, R.D. (2002) Muscle stretching for treatment and prevention of contracture in people with spinal cord injury. *Spinal Cord*, **40**, 1-9.

Hayashi, M., Kamiya, Y., Itoh, H., Higashi, T., Miyazaki, T., Funakoshi, K., Yamashita, N., Goshima, Y., Andoh, T., Yamada, Y. & Goto, T. (2011) Intrathecally administered Sema3A protein attenuates neuropathic pain behavior in rats with chronic constriction injury of the sciatic nerve. *Neuroscience research*, **69**, 17-24.

Hellal, F., Hurtado, A., Ruschel, J., Flynn, K.C., Laskowski, C.J., Umlauf, M., Kapitein, L.C., Strikis, D., Lemmon, V., Bixby, J., Hoogenraad, C.C. & Bradke, F. (2011) Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. *Science*, **331**, 928-931.

Hiebert, G.W., Khodarahmi, K., McGraw, J., Steeves, J.D. & Tetzlaff, W. (2002) Brain-derived neurotrophic factor applied to the motor cortex promotes sprouting of corticospinal fibers but not regeneration into a peripheral nerve transplant. *Journal of neuroscience research*, **69**, 160-168.

Hofstetter, C.P., Holmstrom, N.A., Lilja, J.A., Schweinhardt, P., Hao, J., Spenger, C., Wiesenfeld-Hallin, Z., Kurpad, S.N., Frisen, J. & Olson, L. (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nature neuroscience*, **8**, 346-353.

Hofstetter, C.P., Schwarz, E.J., Hess, D., Widenfalk, J., El Manira, A., Prockop, D.J. & Olson, L. (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 2199-2204.

Hollis, E.R., 2nd, Jamshidi, P., Low, K., Blesch, A. & Tuszynski, M.H. (2009) Induction of corticospinal regeneration by lentiviral trkB-induced Erk activation. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 7215-7220.

Horn, K.P., Busch, S.A., Hawthorne, A.L., van Rooijen, N. & Silver, J. (2008) Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. *J Neurosci*, **28**, 9330-9341.

Houle, J.D., Tom, V.J., Mayes, D., Wagoner, G., Phillips, N. & Silver, J. (2006a) Combining an autologous peripheral nervous system "bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *J Neurosci*, **26**, 7405-7415.

Houle, J.D., Tom, V.J., Mayes, D., Wagoner, G., Phillips, N. & Silver, J. (2006b) Combining an autologous peripheral nervous system "bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 7405-7415.

Huang, E.J. & Reichardt, L.F. (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci*, **24**, 677-736.

Huang, J.K., Phillips, G.R., Roth, A.D., Pedraza, L., Shan, W., Belkaid, W., Mi, S., Fex-Svenningsen, A., Florens, L., Yates, J.R., 3rd & Colman, D.R. (2005) Glial membranes at the node of Ranvier prevent neurite outgrowth. *Science*, **310**, 1813-1817.

Hurlbert, R.J. (2006) Strategies of medical intervention in the management of acute spinal cord injury. *Spine (Phila Pa 1976)*, **31**, S16-21; discussion S36.

Ikeda, H. & Murase, K. (2004) Glial nitric oxide-mediated long-term presynaptic facilitation revealed by optical imaging in rat spinal dorsal horn. *J Neurosci*, **24**, 9888-9896.

Jakeman, L.B. & Reier, P.J. (1991) Axonal projections between fetal spinal cord transplants and the adult rat spinal cord: a neuroanatomical tracing study of local interactions. *The Journal of comparative neurology*, **307**, 311-334.

Jankowski, M.P., Cornuet, P.K., McIlwrath, S., Koerber, H.R. & Albers, K.M. (2006) SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. *Neuroscience*, **143**, 501-514.

Ji, B., Li, M., Wu, W.T., Yick, L.W., Lee, X., Shao, Z., Wang, J., So, K.F., McCoy, J.M., Pepinsky, R.B., Mi, S. & Relton, J.K. (2006) LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Molecular and cellular neurosciences*, **33**, 311-320.

Jiang, S.D., Dai, L.Y. & Jiang, L.S. (2006) Osteoporosis after spinal cord injury. *Osteoporos Int*, **17**, 180-192.

Jones, T.B., McDaniel, E.E. & Popovich, P.G. (2005) Inflammatory-mediated injury and repair in the traumatically injured spinal cord. *Curr Pharm Des*, **11**, 1223-1236.

Josephson, A., Trifunovski, A., Widmer, H.R., Widenfalk, J., Olson, L. & Spenger, C. (2002) Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. *The Journal of comparative neurology*, **453**, 292-304.

Kaneko, S., Iwanami, A., Nakamura, M., Kishino, A., Kikuchi, K., Shibata, S., Okano, H.J., Ikegami, T., Moriya, A., Konishi, O., Nakayama, C., Kumagai, K., Kimura, T., Sato, Y., Goshima, Y., Taniguchi, M., Ito, M., He, Z., Toyama, Y. & Okano, H. (2006) A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. *Nat Med*, **12**, 1380-1389.

Karlsson, A.K. (1999) Autonomic dysreflexia. *Spinal Cord*, **37**, 383-391.

Kawaja, M.D. & Gage, F.H. (1991) Reactive astrocytes are substrates for the growth of adult CNS axons in the presence of elevated levels of nerve growth factor. *Neuron*, **7**, 1019-1030.

Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K. & Steward, O. (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **25**, 4694-4705.

Kim, J.E., Li, S., GrandPre, T., Qiu, D. & Strittmatter, S.M. (2003) Axon regeneration in young adult mice lacking Nogo-A/B. *Neuron*, **38**, 187-199.

Kim, J.E., Liu, B.P., Park, J.H. & Strittmatter, S.M. (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. *Neuron*, **44**, 439-451.

Kirshblum, S.C., Priebe, M.M., Ho, C.H., Scelza, W.M., Chiodo, A.E. & Wuermser, L.A. (2007) Spinal cord injury medicine. 3. Rehabilitation phase after acute spinal cord injury. *Arch Phys Med Rehabil*, **88**, S62-70.

Kobayashi, N.R., Fan, D.P., Giehl, K.M., Bedard, A.M., Wiegand, S.J. & Tetzlaff, W. (1997) BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **17**, 9583-9595.

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

Kromer, L.F. (1987) Nerve growth factor treatment after brain injury prevents neuronal death. *Science*, **235**, 214-216.

Kromer, L.F. & Cornbrooks, C.J. (1985) Transplants of Schwann cell cultures promote axonal regeneration in the adult mammalian brain. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 6330-6334.

La Rosa, G., Conti, A., Cardali, S., Cacciola, F. & Tomasello, F. (2004) Does early decompression improve neurological outcome of spinal cord injured patients? Appraisal of the literature using a meta-analytical approach. *Spinal Cord*, **42**, 503-512.

Lazarov-Spiegler, O., Solomon, A.S., Zeev-Brann, A.B., Hirschberg, D.L., Lavie, V. & Schwartz, M. (1996) Transplantation of activated macrophages overcomes central nervous system regrowth failure. *FASEB J*, **10**, 1296-1302.

Lazo, M.G., Shirazi, P., Sam, M., Giobbie-Hurder, A., Blacconiere, M.J. & Muppidi, M. (2001) Osteoporosis and risk of fracture in men with spinal cord injury. *Spinal Cord*, **39**, 208-214.

Lee, J.K., Geoffroy, C.G., Chan, A.F., Tolentino, K.E., Crawford, M.J., Leal, M.A., Kang, B. & Zheng, B. (2010) Assessing spinal axon regeneration and sprouting in Nogo-, MAG-, and OMgp-deficient mice. *Neuron*, **66**, 663-670.

Lee, R., Kermani, P., Teng, K.K. & Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins. *Science*, **294**, 1945-1948.

Leon, S., Yin, Y., Nguyen, J., Irwin, N. & Benowitz, L.I. (2000) Lens injury stimulates axon regeneration in the mature rat optic nerve. *J Neurosci*, **20**, 4615-4626.

Levi, A.D., Dancausse, H., Li, X., Duncan, S., Horkey, L. & Oliviera, M. (2002) Peripheral nerve grafts promoting central nervous system regeneration after spinal cord injury in the primate. *Journal of neurosurgery*, **96**, 197-205.

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

Liebl, D.J., Morris, C.J., Henkemeyer, M. & Parada, L.F. (2003) mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous system. *Journal of neuroscience research*, **71**, 7-22.

Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., Fouad, K., Mir, A., Rausch, M., Kindler, D., Hamers, F.P. & Schwab, M.E. (2005) Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. *Annals of neurology*, **58**, 706-719.

Liesi, P., Kaakkola, S., Dahl, D. & Vaheri, A. (1984) Laminin is induced in astrocytes of adult brain by injury. *EMBO J*, **3**, 683-686.

Liu, B.P., Fournier, A., GrandPre, T. & Strittmatter, S.M. (2002) Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. *Science*, **297**, 1190-1193.

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B. & He, Z. (2010a) PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature neuroscience*, **13**, 1075-1081.

Liu, K., Tedeschi, A., Park, K.K. & He, Z. (2010b) Neuronal Intrinsic Mechanisms of Axon Regeneration. *Annu Rev Neurosci*.

Liu, X., Hawkes, E., Ishimaru, T., Tran, T. & Sretavan, D.W. (2006) EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 3087-3101.

Low, K., Culbertson, M., Bradke, F., Tessier-Lavigne, M. & Tuszynski, M.H. (2008) Netrin-1 is a novel myelin-associated inhibitor to axon growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **28**, 1099-1108.

Lu, H.Z., Wang, Y.X., Zou, J., Li, Y., Fu, S.L., Jin, J.Q., Hu, J.G. & Lu, P.H. (2010) Differentiation of neural precursor cell-derived oligodendrocyte progenitor cells following transplantation into normal and injured spinal cords. *Differentiation*, **80**, 228-240.

Lu, P., Jones, L.L. & Tuszynski, M.H. (2005) BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. *Experimental neurology*, **191**, 344-360.

Lu, P., Yang, H., Culbertson, M., Graham, L., Roskams, A.J. & Tuszynski, M.H. (2006) Olfactory ensheathing cells do not exhibit unique migratory or axonal growth-promoting properties after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 11120-11130.

Lu, P., Yang, H., Jones, L.L., Filbin, M.T. & Tuszynski, M.H. (2004) Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 6402-6409.

Ma, M., Basso, D.M., Walters, P., Stokes, B.T. & Jakeman, L.B. (2001) Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. *Experimental neurology*, **169**, 239-254.

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

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

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J. & Braun, P.E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron*, **13**, 805-811.

McKinley, W.O., Jackson, A.B., Cardenas, D.D. & DeVivo, M.J. (1999) Long-term medical complications after traumatic spinal cord injury: a regional model systems analysis. *Arch Phys Med Rehabil*, **80**, 1402-1410.

Merli, G.J., Crabbe, S., Paluzzi, R.G. & Fritz, D. (1993) Etiology, incidence, and prevention of deep vein thrombosis in acute spinal cord injury. *Arch Phys Med Rehabil*, **74**, 1199-1205.

Meyer, M., Matsuoka, I., Wetmore, C., Olson, L. & Thoenen, H. (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *J Cell Biol*, **119**, 45-54.

Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., Levesque, M., Allaire, N., Perrin, S., Sands, B., Crowell, T., Cate, R.L., McCoy, J.M. & Pepinsky, R.B. (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nature neuroscience*, **7**, 221-228.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q. & Snider, W.D. (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron*, **19**, 849-861.

Moon, L. & Bunge, M.B. (2005) From animal models to humans: strategies for promoting CNS axon regeneration and recovery of limb function after spinal cord injury. *J Neurol Phys Ther*, **29**, 55-69.

Moon, L.D., Asher, R.A., Rhodes, K.E. & Fawcett, J.W. (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nature neuroscience*, **4**, 465-466.

Moore, S.W., Tessier-Lavigne, M. & Kennedy, T.E. (2007) Netrins and their receptors. *Advances in experimental medicine and biology*, **621**, 17-31.

Moreau-Fauvarque, C., Kumanogoh, A., Camand, E., Jaillard, C., Barbin, G., Boquet, I., Love, C., Jones, E.Y., Kikutani, H., Lubetzki, C., Dusart, I. & Chedotal, A. (2003) The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 9229-9239.

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R. & Filbin, M.T. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron*, **13**, 757-767.

Myers, J., Lee, M. & Kiratli, J. (2007) Cardiovascular disease in spinal cord injury: an overview of prevalence, risk, evaluation, and management. *Am J Phys Med Rehabil*, **86**, 142-152.

Nakamura, Y., Fujita, Y., Ueno, M., Takai, T. & Yamashita, T. (2011) Paired immunoglobulin-like receptor B knockout does not enhance axonal regeneration or locomotor recovery after spinal cord injury. *J Biol Chem*, **286**, 1876-1883.

Neumann, S., Bradke, F., Tessier-Lavigne, M. & Basbaum, A.I. (2002) Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron*, **34**, 885-893.

Neumann, S. & Woolf, C.J. (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron*, **23**, 83-91.

Novikova, L.N., Brohlin, M., Kingham, P.J., Novikov, L.N. & Wiberg, M. (2011) Neuroprotective and growth-promoting effects of bone marrow stromal cells after cervical spinal cord injury in adult rats. *Cytotherapy*.

Oudega, M., Varon, S. & Hagg, T. (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: promoting effects of conditioning lesion and graft predegeneration. *Experimental neurology*, **129**, 194-206.

Paino, C.L. & Bunge, M.B. (1991) Induction of axon growth into Schwann cell implants grafted into lesioned adult rat spinal cord. *Experimental neurology*, **114**, 254-257.

Park, E., Velumian, A.A. & Fehlings, M.G. (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma*, **21**, 754-774.

Park, J.B., Yiu, G., Kaneko, S., Wang, J., Chang, J., He, X.L., Garcia, K.C. & He, Z. (2005) A TNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron*, **45**, 345-351.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M. & He, Z. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*, **322**, 963-966.

Pasterkamp, R.J., Anderson, P.N. & Verhaagen, J. (2001) Peripheral nerve injury fails to induce growth of lesioned ascending dorsal column axons into spinal cord scar tissue expressing the axon repellent Semaphorin3A. *The European journal of neuroscience*, **13**, 457-471.

Pasterkamp, R.J., Giger, R.J., Ruitenberg, M.J., Holtmaat, A.J., De Wit, J., De Winter, F. & Verhaagen, J. (1999) Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Molecular and cellular neurosciences*, **13**, 143-166.

Pearse, D.D., Chatzipanteli, K., Marcillo, A.E., Bunge, M.B. & Dietrich, W.D. (2003) Comparison of iNOS inhibition by antisense and pharmacological inhibitors after spinal cord injury. *J Neuropathol Exp Neurol*, **62**, 1096-1107.

Pearse, D.D., Sanchez, A.R., Pereira, F.C., Andrade, C.M., Puzis, R., Pressman, Y., Golden, K., Kitay, B.M., Blits, B., Wood, P.M. & Bunge, M.B. (2007) Transplantation of Schwann cells and/or olfactory ensheathing glia into the contused spinal cord: Survival, migration, axon association, and functional recovery. *Glia*, **55**, 976-1000.

Perry, V.H., Brown, M.C. & Gordon, S. (1987) The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. *J Exp Med*, **165**, 1218-1223.

Plant, G.W., Currier, P.F., Cuervo, E.P., Bates, M.L., Pressman, Y., Bunge, M.B. & Wood, P.M. (2002) Purified adult ensheathing glia fail to myelinate axons under culture conditions that enable Schwann cells to form myelin. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 6083-6091.

Plunet, W., Kwon, B.K. & Tetzlaff, W. (2002) Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. *Journal of neuroscience research*, **68**, 1-6.

Prinjha, R., Moore, S.E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D.L. & Walsh, F.S. (2000) Inhibitor of neurite outgrowth in humans. *Nature*, **403**, 383-384.

Qian, T., Guo, X., Levi, A.D., Vanni, S., Shebert, R.T. & Sipski, M.L. (2005) High-dose methylprednisolone may cause myopathy in acute spinal cord injury patients. *Spinal Cord*, **43**, 199-203.

Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S. & Filbin, M.T. (2002) Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron*, **34**, 895-903.

Rabchevsky, A.G. & Streit, W.J. (1997) Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. *J Neurosci Res*, **47**, 34-48.

Ramer, L.M., Ramer, M.S. & Steeves, J.D. (2005) Setting the stage for functional repair of spinal cord injuries: a cast of thousands. *Spinal Cord*, **43**, 134-161.

Ramón y Cajal, S., DeFelipe, J. & Jones, E.G. (1991) *Cajal's degeneration and regeneration of the nervous system*. Oxford University Press, New York.

Reier, P.J., Bregman, B.S. & Wujek, J.R. (1986) Intraspinal transplantation of embryonic spinal cord tissue in neonatal and adult rats. *The Journal of comparative neurology*, **247**, 275-296.

Reza, J.N., Gavazzi, I. & Cohen, J. (1999) Neuropilin-1 is expressed on adult mammalian dorsal root ganglion neurons and mediates semaphorin3a/collapsin-1-induced growth cone collapse by small diameter sensory afferents. *Molecular and cellular neurosciences*, **14**, 317-326.

Richardson, P.M. & Issa, V.M. (1984) Peripheral injury enhances central regeneration of primary sensory neurones. *Nature*, **309**, 791-793.

Roberts, I., Yates, D., Sandercock, P., Farrell, B., Wasserberg, J., Lomas, G., Cottingham, R., Svoboda, P., Brayley, N., Mazairac, G., Laloe, V., Munoz-Sanchez, A., Arango, M., Hartzenberg, B., Khamis, H., Yutthakasemsunt, S., Komolafe, E., Olldashi, F., Yadav, Y., Murillo-Cabezas, F., Shakur, H. & Edwards, P. (2004) Effect of intravenous corticosteroids on death within 14 days in 10008 adults with clinically significant head injury (MRC CRASH trial): randomised placebo-controlled trial. *Lancet*, **364**, 1321-1328.

Rossignol, S., Schwab, M., Schwartz, M. & Fehlings, M.G. (2007) Spinal cord injury: time to move? *J Neurosci*, **27**, 11782-11792.

Saito, A., Maier, C.M., Narasimhan, P., Nishi, T., Song, Y.S., Yu, F., Liu, J., Lee, Y.S., Nito, C., Kamada, H., Dodd, R.L., Hsieh, L.B., Hassid, B., Kim, E.E., Gonzalez, M. & Chan, P.H. (2005) Oxidative stress and neuronal death/survival signaling in cerebral ischemia. *Mol Neurobiol*, **31**, 105-116.

Sasaki, M., Hains, B.C., Lankford, K.L., Waxman, S.G. & Kocsis, J.D. (2006) Protection of corticospinal tract neurons after dorsal spinal cord transection and engraftment of olfactory ensheathing cells. *Glia*, **53**, 352-359.

Schnell, L. & Schwab, M.E. (1993) Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord. *The European journal of neuroscience*, **5**, 1156-1171.

Schreyer, D.J. & Skene, J.H. (1993) Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *J Neurobiol*, **24**, 959-970.

Schwab, M.E. & Bartholdi, D. (1996) Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev*, **76**, 319-370.

Seijffers, R., Mills, C.D. & Woolf, C.J. (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J Neurosci*, **27**, 7911-7920.

Selmaj, K.W. & Raine, C.S. (1988) Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol*, **23**, 339-346.

Sengottuvel, V., Leibinger, M., Pfreimer, M., Andreadaki, A. & Fischer, D. (2011) Taxol facilitates axon regeneration in the mature CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **31**, 2688-2699.

Shao, Z., Browning, J.L., Lee, X., Scott, M.L., Shulga-Morskaya, S., Allaire, N., Thill, G., Levesque, M., Sah, D., McCoy, J.M., Murray, B., Jung, V., Pepinsky, R.B. & Mi, S. (2005) TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. *Neuron*, **45**, 353-359.

Sharp, J. & Keirstead, H.S. (2009) Stem cell-based cell replacement strategies for the central nervous system. *Neuroscience letters*, **456**, 107-111.

Silver, J. & Miller, J.H. (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci*, **5**, 146-156.

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

Siroky, M.B. (2002) Pathogenesis of bacteriuria and infection in the spinal cord injured patient. *Am J Med*, **113 Suppl 1A**, 67S-79S.

Smith-Thomas, L.C., Fok-Seang, J., Stevens, J., Du, J.S., Muir, E., Faissner, A., Geller, H.M., Rogers, J.H. & Fawcett, J.W. (1994) An inhibitor of neurite outgrowth produced by astrocytes. *J Cell Sci*, **107 (Pt 6)**, 1687-1695.

Smith, D.S. & Skene, J.H. (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **17**, 646-658.

Snow, D.M., Lemmon, V., Carrino, D.A., Caplan, A.I. & Silver, J. (1990) Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Exp Neurol*, **109**, 111-130.

Snow, D.M., Watanabe, M., Letourneau, P.C. & Silver, J. (1991) A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. *Development*, **113**, 1473-1485.

Sofroniew, M.V. (2005) Reactive astrocytes in neural repair and protection. *Neuroscientist*, **11**, 400-407.

Song, X.Y., Zhong, J.H., Wang, X. & Zhou, X.F. (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 542-546.

Sorensen, J.C., Grabowski, M., Zimmer, J. & Johansson, B.B. (1996) Fetal neocortical tissue blocks implanted in brain infarcts of adult rats interconnect with the host brain. *Experimental neurology*, **138**, 227-235.

Stegmuller, J., Konishi, Y., Huynh, M.A., Yuan, Z., Dibacco, S. & Bonni, A. (2006) Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron*, **50**, 389-400.

Steinmetz, M.P., Horn, K.P., Tom, V.J., Miller, J.H., Busch, S.A., Nair, D., Silver, D.J. & Silver, J. (2005) Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. *J Neurosci*, **25**, 8066-8076.

Stevens, R.D., Bhardwaj, A., Kirsch, J.R. & Mirski, M.A. (2003) Critical care and perioperative management in traumatic spinal cord injury. *J Neurosurg Anesthesiol*, **15**, 215-229.

Steward, O., Zheng, B., Banos, K. & Yee, K.M. (2007) Response to: Kim et al., "axon regeneration in young adult mice lacking Nogo-A/B." Neuron 38, 187-199. *Neuron*, **54**, 191-195.

Stone, J.M., Nino-Murcia, M., Wolfe, V.A. & Perkash, I. (1990) Chronic gastrointestinal problems in spinal cord injury patients: a prospective analysis. *Am J Gastroenterol*, **85**, 1114-1119.

Suh, H.I., Min, J., Choi, K.H., Kim, S.W., Kim, K.S. & Jeon, S.R. (2011) Axonal regeneration effects of Wnt3a-secreting fibroblast transplantation in spinal cord-injured rats. *Acta Neurochir (Wien)*, **153**, 1003-1010.

Sundberg, M., Skottman, H., Suuronen, R. & Narkilahti, S. (2010) Production and isolation of NG2+ oligodendrocyte precursors from human embryonic stem cells in defined serum-free medium. *Stem Cell Res*, **5**, 91-103.

Tang, X.Q., Tanelian, D.L. & Smith, G.M. (2004) Semaphorin3A inhibits nerve growth factor-induced sprouting of nociceptive afferents in adult rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 819-827.

Taylor, L., Jones, L., Tuszynski, M.H. & Blesch, A. (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 9713-9721.

Teasell, R.W., Mehta, S., Aubut, J.L., Ashe, M.C., Sequeira, K., Macaluso, S. & Tu, L. (2010) A systematic review of the therapeutic interventions for heterotopic ossification after spinal cord injury. *Spinal Cord*, **48**, 512-521.

Thanos, S. (1992) Sick photoreceptors attract activated microglia from the ganglion cell layer: a model to study the inflammatory cascades in rats with inherited retinal dystrophy. *Brain Res*, **588**, 21-28.

Tobias, C.A., Shumsky, J.S., Shibata, M., Tuszynski, M.H., Fischer, I., Tessler, A. & Murray, M. (2003) Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. *Experimental neurology*, **184**, 97-113.

Tsuji, O., Miura, K., Okada, Y., Fujiyoshi, K., Mukaino, M., Nagoshi, N., Kitamura, K., Kumagai, G., Nishino, M., Tomisato, S., Higashi, H., Nagai, T., Katoh, H., Kohda, K., Matsuzaki, Y., Yuzaki, M., Ikeda, E., Toyama, Y., Nakamura, M., Yamanaka, S. & Okano, H. (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 12704-12709.

Turnley, A.M. & Bartlett, P.F. (1998) MAG and MOG enhance neurite outgrowth of embryonic mouse spinal cord neurons. *Neuroreport*, **9**, 1987-1990.

Tuszynski, M.H. (2007) Rebuilding the brain: resurgence of fetal grafting. *Nature neuroscience*, **10**, 1229-1230.

Tuszynski, M.H., Gabriel, K., Gage, F.H., Suhr, S., Meyer, S. & Rosetti, A. (1996) Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. *Experimental neurology*, **137**, 157-173.

Tuszynski, M.H., Grill, R., Jones, L.L., Brant, A., Blesch, A., Low, K., Lacroix, S. & Lu, P. (2003) NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection. *Experimental neurology*, **181**, 47-56.

Tuszynski, M.H., Peterson, D.A., Ray, J., Baird, A., Nakahara, Y. & Gage, F.H. (1994) Fibroblasts genetically modified to produce nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. *Experimental neurology*, **126**, 1-14.

Tuszynski, M.H., Weidner, N., McCormack, M., Miller, I., Powell, H. & Conner, J. (1998) Grafts of genetically modified Schwann cells to the spinal cord: survival, axon growth, and myelination. *Cell Transplant*, **7**, 187-196.

Vaccaro, A.R., Daugherty, R.J., Sheehan, T.P., Dante, S.J., Cotler, J.M., Balderston, R.A., Herbison, G.J. & Northrup, B.E. (1997) Neurologic outcome of early versus late surgery for cervical spinal cord injury. *Spine (Phila Pa 1976)*, **22**, 2609-2613.

van Kuijk, A.A., Geurts, A.C. & van Kuppevelt, H.J. (2002) Neurogenic heterotopic ossification in spinal cord injury. *Spinal Cord*, **40**, 313-326.

Vaquero, J. & Zurita, M. (2011) Functional recovery after severe CNS trauma: current perspectives for cell therapy with bone marrow stromal cells. *Progress in neurobiology*, **93**, 341-349.

Venkatesh, K., Chivatakarn, O., Lee, H., Joshi, P.S., Kantor, D.B., Newman, B.A., Mage, R., Rader, C. & Giger, R.J. (2005) The Nogo-66 receptor homolog NgR2 is a

sialic acid-dependent receptor selective for myelin-associated glycoprotein. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **25**, 808-822.

Vroemen, M., Aigner, L., Winkler, J. & Weidner, N. (2003) Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. *The European journal of neuroscience*, **18**, 743-751.

Wang, K.C., Kim, J.A., Sivasankaran, R., Segal, R. & He, Z. (2002a) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature*, **420**, 74-78.

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L. & He, Z. (2002b) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature*, **417**, 941-944.

Wilkins, A., Chandran, S. & Compston, A. (2001) A role for oligodendrocyte-derived IGF-1 in trophic support of cortical neurons. *Glia*, **36**, 48-57.

Wilkins, A., Majed, H., Layfield, R., Compston, A. & Chandran, S. (2003) Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 4967-4974.

Willson, C.A., Irizarry-Ramirez, M., Gaskins, H.E., Cruz-Orengo, L., Figueroa, J.D., Whittemore, S.R. & Miranda, J.D. (2002) Upregulation of EphA receptor expression in the injured adult rat spinal cord. *Cell Transplant*, **11**, 229-239.

Wong, S.T., Henley, J.R., Kanning, K.C., Huang, K.H., Bothwell, M. & Poo, M.M. (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nature neuroscience*, **5**, 1302-1308.

Wuermser, L.A., Ho, C.H., Chiodo, A.E., Priebe, M.M., Kirshblum, S.C. & Scelza, W.M. (2007) Spinal cord injury medicine. 2. Acute care management of traumatic and nontraumatic injury. *Arch Phys Med Rehabil*, **88**, S55-61.

Xie, F. & Zheng, B. (2008) White matter inhibitors in CNS axon regeneration failure. *Experimental neurology*, **209**, 302-312.

Yamaguchi, Y. (2000) Lecticans: organizers of the brain extracellular matrix. *Cell Mol Life Sci*, **57**, 276-289.

Yamashita, T., Higuchi, H. & Tohyama, M. (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J Cell Biol*, **157**, 565-570.

Yarkony, G.M. (1994) Pressure ulcers: a review. *Arch Phys Med Rehabil*, **75**, 908-917.

Yi, J.J., Barnes, A.P., Hand, R., Polleux, F. & Ehlers, M.D. (2010) TGF-beta signaling specifies axons during brain development. *Cell*, **142**, 144-157.

Yin, Y., Cui, Q., Li, Y., Irwin, N., Fischer, D., Harvey, A.R. & Benowitz, L.I. (2003) Macrophage-derived factors stimulate optic nerve regeneration. *J Neurosci*, **23**, 2284-2293.

Yin, Y., Henzl, M.T., Lorber, B., Nakazawa, T., Thomas, T.T., Jiang, F., Langer, R. & Benowitz, L.I. (2006) Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nature neuroscience*, **9**, 843-852.

Yune, T.Y., Chang, M.J., Kim, S.J., Lee, Y.B., Shin, S.W., Rhim, H., Kim, Y.C., Shin, M.L., Oh, Y.J., Han, C.T., Markelonis, G.J. & Oh, T.H. (2003) Increased production of tumor necrosis factor-alpha induces apoptosis after traumatic spinal cord injury in rats. *J Neurotrauma*, **20**, 207-219.

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

Zou, H., Ho, C., Wong, K. & Tessier-Lavigne, M. (2009) Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci*, **29**, 7116-7123.

CHAPTER 2

SnoN Facilitates Axonal Regeneration After Spinal Cord Injury

Abstract

CNS neurons in the vicinity of an injury encounter an environment dramatically altered by the release of factors induced by trauma, including TGF-β1, a member of the TGF-β superfamily. We now show that upregulation of TGF-β1 directly inhibits neuronal process outgrowth and leads to intraneuronal degradation of the developmental axon-specific growth-associated molecule SnoN. Overexpression of SnoN *in vitro* promotes axonal growth and overcomes TGF-β1 induced inhibition. Furthermore, *in vivo* overexpression of SnoN enhances central sensory axonal regeneration following spinal cord injury. Thus, neuronal TGF-β signaling is a novel negative modulator of axonal growth after injury; targeting this pathway through overexpression of the specific axonal growth-enhancing molecule SnoN promotes growth of injured adult axons.

Introduction

Several mechanisms contribute to the failure of axonal regeneration in the adult central nervous system, including factors both intrinsic and extrinsic to the neuron. The importance of neuron-intrinsic mechanisms in regeneration failure are illustrated by the effects of a "conditioning" lesion, wherein injury to the peripheral process of a dorsal root ganglion neuron substantially enhances regeneration of the central process of the same neuron after spinal cord injury (Neumann & Woolf, 1999; Alto *et al.*, 2009), mediated in part by upregulation of regeneration-associated genes in the conditioned neuron (Cai *et al.*, 2002; Gao *et al.*, 2004; Zou *et al.*, 2009). However, the full set of intrinsic neuronal mechanisms that underlie central axonal regeneration failure remain poorly understood,

Studies of neural development may provide insight into intrinsic neuronal mechanisms influencing axonal growth, since developing axons exhibit an extensive growth capacity. Recent studies have revealed a previously unrecognized role for members of the transforming growth factor-beta (TGF-β) superfamily in developmentally-regulated axon growth. Historically, bone morphogenetic proteins (BMPs) have been the most prominent member of the TGF-β superfamily associated with axon growth and guidance (Lee *et al.*, 1998; Charron & Tessier-Lavigne, 2007; Zou *et al.*, 2009). However, recent findings suggest potential dual roles of TGF-ß signaling in developmental regulation of axon growth: while TGF-ß is necessary for specification of the developing axon (Asher *et al.*, 2000; Lagord *et al.*, 2002; Yi *et al.*, 2010), it also

activates signaling that degrades neuronal mechanisms that support axonal growth. That is, TGF-β ligands convey their effects through ligand-induced clustering of TGF-βRI and TGF-βRII serine/threonine kinase receptors and concomitant phosphorylation of SMAD2/3 (Massague, 2000). Phosphorylated SMAD2/3 alters transcription, thereby facilitating the degradation of an integral molecular signal in the process of axonal growth, the transcriptional regulator SnoN (Stegmuller *et al.*, 2008). Indeed, over-expression of a mutant form of SnoN that is resistant to ubiquitin-mediated degradation promotes axonal growth in developing neurons (Stegmuller *et al.*, 2006). Thus, given its important role in axonal specification and growth during neural development, TGFß is a compelling novel candidate mechanism for examining the intrinsic growth state of the injured adult axon. However, this molecule could act as either a positive or negative effector of the neuronal growth state. Moreover, SnoN represents an intriguing target for potentially modifying the intrinsic growth state of the injured central axon in a positive manner.

Hence, we examined the role of TGFβ signaling and SnoN over-expression in axonal outgrowth in vitro, and in vivo after adult spinal cord injury. We find that TGF-ß signaling is upregulated after spinal cord injury, and this activation is associated with axonal growth inhibition *in vitro*. Consistent with the role of SnoN in axonal growth during development, over-expression of SnoN *in vitro* and *in vivo* is sufficient to overcome TGF-β mediated inhibition and to promote significant central axonal regeneration after spinal cord injury.

Results

TGF-β Inhibits Outgrowth of Adult DRG Neurons

To determine whether neurite outgrowth from adult DRG neurons in vitro is sensitive to TGF-β signaling, we isolated adult DRG neurons from F344 rats and treated the cultures with TGF-β1, 2, or 3 (Fig 1). TGF-β1 significantly inhibited adult DRG neurite outgrowth (Fig 1), reducing the length of the longest neurite at concentrations of 10, 50, and 100 ng/mL ($P<0.05$) and inhibiting total neurite outgrowth at concentrations of 50 and 100 ng/mL ($P<0.05$). TGF-β1 did not significantly affect the number of processes emerging from the neuron, indicating TGF-ß does not influence neurite emergence or branching from the soma (Fig. 1). TGF-β3 also increased longest neurite length and total neurite outgrowth but did not affect the number of neuritic processes compared to untreated controls (Suppl Fig. 1). In contrast, TGF-β2 had no effect on longest neurite length, total neurite outgrowth, or number of processes (Suppl Fig 1). These findings indicate that TGF-β signaling inhibits neurite outgrowth from adult neurons but does not reduce neurite initiation (because the number of neuritic processes emerging from the neuron is stable).

We next determined whether neurons are differentially responsive to TGF-β signaling based on their intrinsic growth state, using conditioning lesions of the sciatic nerve (Alto *et al.*, 2009). We isolated and cultured adult neurons from L4 and L5 DRGs

peripherally preconditioned by sciatic nerve crush 7 days earlier. Cultures were treated with TGF-β isoforms as above and neurite outgrowth was quantified. Conditioned DRG neurons demonstrated significantly reduced sensitivity to TGF-β1 after conditioning: doses of 10 and 50ng/ml of TGF-ß1 no longer inhibited neurite outgrowth on measures of longest neurite and total neuritic length (Fig. 2). Similar effects were seen with TGF-ß3 (Suppl Fig 2). Thus, modulation of the neuron-intrinsic growth state has the capacity to overcome inhibitory effects of inhibitory TGF-β1 signaling.

TGF-β1 Inhibition of Outgrowth is Associated with SnoN Degradation

We next determined whether activation of TGF-β signaling proceeded along the same canonical pathways of SMAD2 phosphroylation and SnoN ubiquination in adult neurons that are observed in developing systems (Bonni *et al.*, 2001; Stroschein *et al.*, 2001; Wan *et al.*, 2001). Adult DRG neurons were electroporated with GFP, wild-type SnoN, or a variant of SnoN with a mutated destruction box motif (SnoN-D-box mutation) rendering the molecule resistant to degradation (Stroschein *et al.*, 2001). Adult neurons were again cultured in the presence or absence of TGF-β1, cell lysates were collected and western blots were performed to assess levels of SnoN protein and SMAD2 phosphorylation. Upon stimulation with TGF-β1, levels of SMAD2 phosphorylation in adult DRG neurons significantly increased compared to non-stimulated controls ($P<0.05$; Fig 3). TGF-β1 treatment significantly reduced SnoN levels compared to non-stimulated controls ($P<0.05$; Fig. 3). In contrast, neurons expressing the SnoN D-box mutation did not exhibit reductions in SnoN levels after TGF-ß1 stimulation (Fig 3). These findings

indicate that adult neurons respond to TGF-β1 utilizing known developmental canonical signaling mechanisms, including SMAD2 phosphorylation and SnoN degradation.

Having established that TGF-β1 signaling in adult neurons proceeds through canonical pathways and that one of the consequences of SMAD2 activation appears to be degradation of SnoN, we determined whether the overexpression of SnoN or the SnoN-D-box mutation is sufficient to overcome TGF-β1 inhibition in vitro. Adult DRGs neurons were electroporated with GFP, SnoN, or the SnoN D-box mutation and treated with TGF-β1 (50 ng/ml). Overexpression of SnoN in control DRG neurons (without exposure to TGF-ß1) significantly increased growth (Fig 3, $P<0.05$), while overexpression of SnoN-D-box mutation further increased neurite growth ($P<0.05$). Further, overexpression of SnoN and SnoN-D-box mutation overcame TGF-ß1-induced inhibition of neurite outgrowth (Fig 3), These findings indicate both that SnoN expression alone is sufficient to enhance neurite growth in vitro, and that SnoN overcomes TGF-ß1- inhibition.

Injury Activates Neuronal TGF-β1 Signaling *In Vivo*

To examine the potential relevance of SnoN-based interventions for the treatment of spinal cord injury, TGF-β1 must be released at sites of injury and activate canonical neuronal TGF-β1 signaling. Thus, we performed C3 dorsal column spinal cord lesions in adult F344 rats and examined release of TGF-β1 ligand at the injury site 1 hour, 1 day, or

7 days following injury. TGF-β1 immunoreactivity increased by 1 hour post-injury in the peri-lesioned tissue and remained persistently elevated for at least 7 days (Fig. 4). Regions of increased expression included the dorsal funiculus, which contains transected somatosensory axons arising from neuronal somata in the DRG. Dissection of DRG neurons at the same post-injury time points revealed increases in phosphorylated SMAD2 beginning 1 hour and persisting through 7 days post-lesion, by Western blot (Fig. 4). Thus, TGF-ß1 is expressed at in vivo sites of SCI and activates canonical signaling in the neuronal somata of injured neurons.

SnoN Promotes Axonal Regeneration After Spinal Cord Injury

We next examined the clinical relevance of SnoN-based therapies for spinal cord injury. These in vivo injury studies used the SnoN-D-box mutation, given its more potent effects on neurite outgrowth in vitro (Fig 4). Adeno-associated virus – serotype 6 (AAV6) expressing the SnoN-D-box mutation was injected into L4 and L5 DRG neurons in 12 adult Fischer 344 rats, and axonal regeneration was assessed 4 weeks after placement of a C3 dorsal column spinal cord lesion (Alto *et al.*, 2009; Kadoya *et al.*, 2009). Comparison was made to 11 C3-lesioned controls that received L4 and L5 intraganglionic injections of AAV6-GFP, and to 4 animals that received C3 lesions only.

Axons require a cell matrix in the lesion site to support axonal regeneration, since axons cannot regenerate into a cystic lesion site. Accordingly, all groups received implants of syngenic bone marrow stromal cells, which ordinarily support minimal amounts of

axonal growth (Alto *et al.*, 2009; Kadoya *et al.*, 2009). One month after spinal cord injury, animals treated with the SnoN-D-box mutation showed a significant, 2-fold enhancement of axonal regeneration into the lesion site ($P<0.05$; Fig 5). A relatively small percentage of CTB labeled neurons expresesd GFP (25.4±2.7%) or the SnoN-D-box mutation (7.9±1.2%), suggesting a potential underestimate of the capacity for SnoN overexpression to facilitate axon regeneration (Fig. 5). Assessment of in vivo axonal regeneration must ensure that regenerating axons are not mistaken for spared axons; accordingly, examination of the spinal cord rostral to the lesion site showed an absence of CTB-labeled axons, indicating lesion completeness in all subjects. Further, spared axons would not be located in the ectopic environment of the cell graft in the lesion site (Fig. 5), indicating that axons truly regenerated. Thus, axonal regeneration after spinal cord injury can be promoted by overexpression of SnoN.

Discussion

Findings of this study reveal for the first time a direct neuronal effect of TGF-β signaling on axonal growth and regeneration in the adult nervous system. Specifically, we find that TGF-ß is expressed at sites of spinal cord injury and activates canonical neuronal signaling resulting in phosphorylation of SMAD2 and degradation of SnoN, a transcription factor that specifically regulates axon growth. Intraneuronal overexpression of SnoN is sufficient to overcome TGF-ß-mediated inhibition *in vitro* and enhances axonal regeneration after spinal cord injury. Thus, we identify a novel target mechanism for enhancing central axonal regeneration.

Few studies have investigated axon-specific intrinsic mechanisms to promote regeneration following injury. Pursuing this level of specificity is logical, as nonspecific stimulation of growth from both the axonal and dendritic compartments might increase the potential to form aberrant connections and result in dysfunctional outcomes. SnoN is such a developmentally-regulated molecule, a transcription factor that exhibits a specific effect on axonal outgrowth in postnatal neurons (Konishi *et al.*, 2004; Stegmuller *et al.*, 2006). We now find that this axonal-specific regulator of axonal outgrowth during development also enhances neurite outgrowth in adult DRG neurons, and enhances central axonal regeneration in the injured spinal cord. *In vivo*, SnoN overexpression significantly enhances axonal regeneration into a spinal cord lesion site.

Results of this study also reveal for the first time a direct neuronal influence of TGF-ß signaling on axonal response to injury. Prior studies described effects of TGF-β following CNS injury on glial scar formation, angiogenesis, and immune cell recruitment (Moon & Fawcett, 2001; Schachtrup *et al.*, 2010), but did not describe a direct effect on neuronal signaling and growth. Our studies reveal expression of TGF-ß in sites of spinal cord injury; TGF-ß directly activates SMAD2 in adult DRG neurons and is associated with SnoN degradation, revealing a direct neuronal role for this pathway in inhibiting the growth of injured adult neurons. By targeting the neuron-intrinsic aspects of TGF-β signaling, it may be possible to overcome neuron-intrinsic inhibitory effects on axonal growth while retaining potentially beneficial effects of TGF-ß in reducing secondary damage around sites of SCI.

Experimental Procedures

DRG Culture and Quantification

DRGs were dissected from adult female Fisher 344 rats weighing 150-165 g, digested in 0.25% collagenase type XI and 5ug/mL dispase, and triturated. For transfections, 1 million cells and 10 ug of DNA were used according to Nucleofactor (Lonza) protocols. Plasmids consisted of a dual promoter system which expressed either GFP, GFP-SnoN, or GFP-SnoN-D-box mutation driven by a hybrid chick beta actin – minimal CMV promoter and the reporter gene copGFP driven by an EF1-α promoter. Cells were plated on cell culture plates coated with poly-L-lysine (20ug/mL) and laminin (0.5ug/mL) and cultured in media supplemented with TGF-β1, 2, or 3 partially changed every other day. Cells were fixed with 4% PFA after four days in vitro and stained with βIII-tubulin (Promega), NF200 (abcam), and copGFP (Evrogen). Images were acquired using an ImageXpress Micro (Molecular Devices) and analyzed using the neurite outgrowth module of the MetaXpress software package. For quantification of transfected neurons, only neurons expressing the copGFP reporter were analyzed. All experiments were run in at least triplicate.

Protein Isolation and Western Blot Analysis

Protein was extracted from DRG cultures or from adult L4 and L5 DRGs using RIPA buffer. Protein concentration was determined using Bio-Rad Protein Assay and 20 µg was resolved on a 4-12% SDS-PAGE gel (Invitrogen). The gel was transferred to 0.45

μm PVDF membranes (Millipore), blocked with 5% nonfat milk, washed with 0.05% Tween-20 in PBS, and incubated with anti-pSMAD2 (Cell Signaling), anti-SMAD2 (Cell Signaling), or anti-SnoN (Cell Signaling). Blots were washed and incubated with horseradish peroxidase conjugated secondaries and visualized and analyzed using chemiluminescence on a Bio-Rad GelDoc. Blots were then stripped and reblotted with anti-β-actin (Cell Signaling).

Surgeries and Tissue Processing

Adult female Fisher rats weighing 150-165 g were deeply anesthetized with xylazine, ketamine, and acepromazine for surgeries. Peripheral conditioning lesions were made using jeweler's forceps to compress the exposed sciatic nerve at midthigh for 15 s. For intraganglionic injections, the vertebrate overlaying the L4 and L5 DRGs were removed and 1 μL per DRG of AAV6 (~$1x10^{12}$ infectious units/mL) was injected into the L4 and L5 DRGs bilaterally using a pulled glass micropipette and picospritzer. Great care was taken to avoid damage to the injected DRG as this might "condition" the neurons. C3 dorsal column lesions were performed using a tungsten wire knife. MSCs were isolated as described previously (Hofstetter *et al.*, 2002) and grafted into acute spinal cord dorsal column lesions using fine glass micropipettes and a picospritzer at low pressue. Dorsal column sensory axons were traced by CTB injections (2 μL, 1% solution) into the sciatic nerve 3 days before perfusion. Animals were perfused with 4% PFA. 30 μm thick spinal cord sections were immunohistochemically labeled for CTB and GFAP. 10 μm thick

sections of L4 and L5 DRGs were immunohistochemically labeled for GFP, CTB, and βIII-tubulin.

Axonal Quantification

Axon numbers were quantified using 1-in-6 sections from sagitally sectioned spinal cords within the lesion site. CTB labeled axons were visualized by light level immunohistochemistry using diaminobenzidine (0.05%) and nickel chloride (0.04%) as chromagens. The same sections were then processed for GFAP using fluorescence. Lesion borders were determined using GFAP immunoreactivity. An Olympus BX60 microscope equipped with an a motorized stage and stage controller (MAC2002-XYZ, Ludl Electronic Products, Hawthorne, NY) and StereoInvestigator software (MicroBrightField, Colchester, VT) were used to draw the lesion boundaries and a vertical bisecting the cell graft under low magnification (4× objective). Axons were visualized under high magnification (60× objective) and only axons intersecting the vertical line were scored. Total axon numbers were estimated by multiplying the count by number of sections.

Acknowledgements

Supported by the Veterans Administration, NIH (NS049881 and NS42291), the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, and the Bernard and Anne Spitzer Charitable Trust.

References

Alto, L.T., Havton, L.A., Conner, J.M., Hollis Ii, E.R., Blesch, A. & Tuszynski, M.H. (2009) Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. *Nature neuroscience*, **12**, 1106-1113.

Asher, R.A., Morgenstern, D.A., Fidler, P.S., Adcock, K.H., Oohira, A., Braistead, J.E., Levine, J.M., Margolis, R.U., Rogers, J.H. & Fawcett, J.W. (2000) Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. *J Neurosci*, **20**, 2427-2438.

Bonni, S., Wang, H.R., Causing, C.G., Kavsak, P., Stroschein, S.L., Luo, K. & Wrana, J.L. (2001) TGF-beta induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. *Nat Cell Biol*, **3**, 587-595.

Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R.R. & Filbin, M.T. (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. *Neuron*, **35**, 711-719.

Charron, F. & Tessier-Lavigne, M. (2007) The Hedgehog, TGF-beta/BMP and Wnt families of morphogens in axon guidance. *Adv Exp Med Biol*, **621**, 116-133.

Gao, Y., Deng, K., Hou, J., Bryson, J.B., Barco, A., Nikulina, E., Spencer, T., Mellado, W., Kandel, E.R. & Filbin, M.T. (2004) Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. *Neuron*, **44**, 609-621.

Hofstetter, C.P., Schwarz, E.J., Hess, D., Widenfalk, J., El Manira, A., Prockop, D.J. & Olson, L. (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 2199-2204.

Kadoya, K., Tsukada, S., Lu, P., Coppola, G., Geschwind, D., Filbin, M.T., Blesch, A. & Tuszynski, M.H. (2009) Combined intrinsic and extrinsic neuronal mechanisms facilitate bridging axonal regeneration one year after spinal cord injury. *Neuron*, **64**, 165-172.

Konishi, Y., Stegmuller, J., Matsuda, T., Bonni, S. & Bonni, A. (2004) Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science*, **303**, 1026-1030.

Lagord, C., Berry, M. & Logan, A. (2002) Expression of TGFbeta2 but not TGFbeta1 correlates with the deposition of scar tissue in the lesioned spinal cord. *Mol Cell Neurosci*, **20**, 69-92.

Lee, K.J., Mendelsohn, M. & Jessell, T.M. (1998) Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev*, **12**, 3394-3407.

Massague, J. (2000) How cells read TGF-beta signals. *Nat Rev Mol Cell Biol*, **1**, 169-178.

Moon, L.D. & Fawcett, J.W. (2001) Reduction in CNS scar formation without concomitant increase in axon regeneration following treatment of adult rat brain with a combination of antibodies to TGFbeta1 and beta2. *Eur J Neurosci*, **14**, 1667-1677.

Neumann, S. & Woolf, C.J. (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron*, **23**, 83-91.

Schachtrup, C., Ryu, J.K., Helmrick, M.J., Vagena, E., Galanakis, D.K., Degen, J.L., Margolis, R.U. & Akassoglou, K. (2010) Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J Neurosci*, **30**, 5843-5854.

Stegmuller, J., Huynh, M.A., Yuan, Z., Konishi, Y. & Bonni, A. (2008) TGFbeta-Smad2 signaling regulates the Cdh1-APC/SnoN pathway of axonal morphogenesis. *J Neurosci*, **28**, 1961-1969.

Stegmuller, J., Konishi, Y., Huynh, M.A., Yuan, Z., Dibacco, S. & Bonni, A. (2006) Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron*, **50**, 389-400.

Stroschein, S.L., Bonni, S., Wrana, J.L. & Luo, K. (2001) Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. *Genes & development*, **15**, 2822-2836.

Wan, Y., Liu, X. & Kirschner, M.W. (2001) The anaphase-promoting complex mediates TGF-beta signaling by targeting SnoN for destruction. *Mol Cell*, **8**, 1027-1039.

Yi, J.J., Barnes, A.P., Hand, R., Polleux, F. & Ehlers, M.D. (2010) TGF-beta signaling specifies axons during brain development. *Cell*, **142**, 144-157.

Zou, H., Ho, C., Wong, K. & Tessier-Lavigne, M. (2009) Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci*, **29**, 7116-7123.

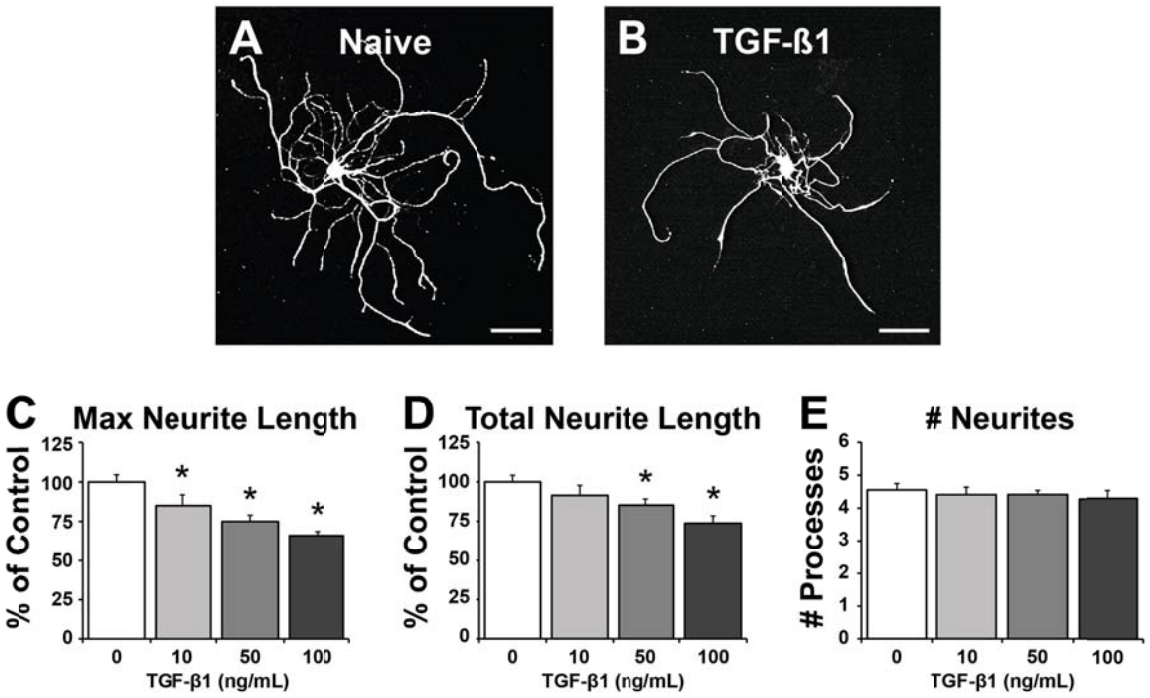


Figure 2.1: TGF-β1 Inhibits Outgrowth of Adult DRG Neurons

Adult DRG neurons plated on poly-L-lysine and laminin. (**A**) Adult DRG neurons cultured for 4 days extend multiple processes. (**B**) Adult DRG neurons treated with 50 ng/mL TGF-β1 exhibit reduced maximal and total neurite length, quantified in **C-E**. (**C**) Longest neurite (ANOVA $p<0.01$; *, post hoc Fisher's $p<0.05$). (**D**) Total neurite length (ANOVA $p<0.01$; *, post hoc Fisher's $p<0.05$). (**E**) TGF-β1 did not affect the number of neurites emerging from the adult DRG neuron. Scale bar A, B: 100μm.

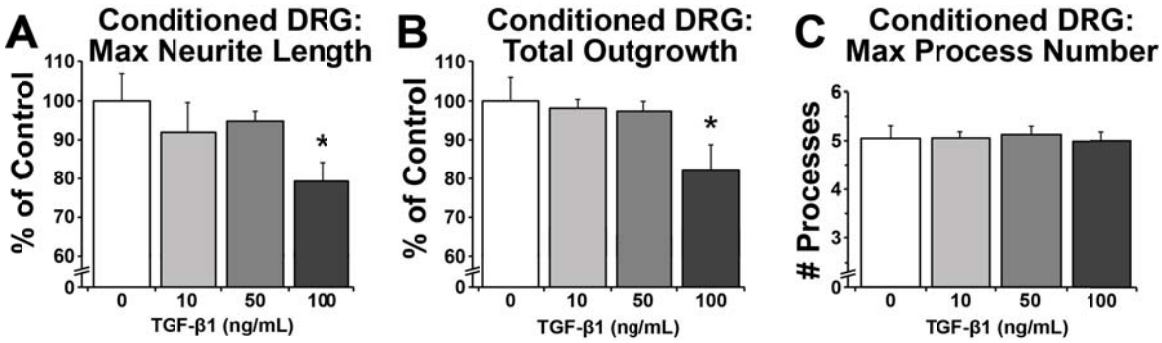
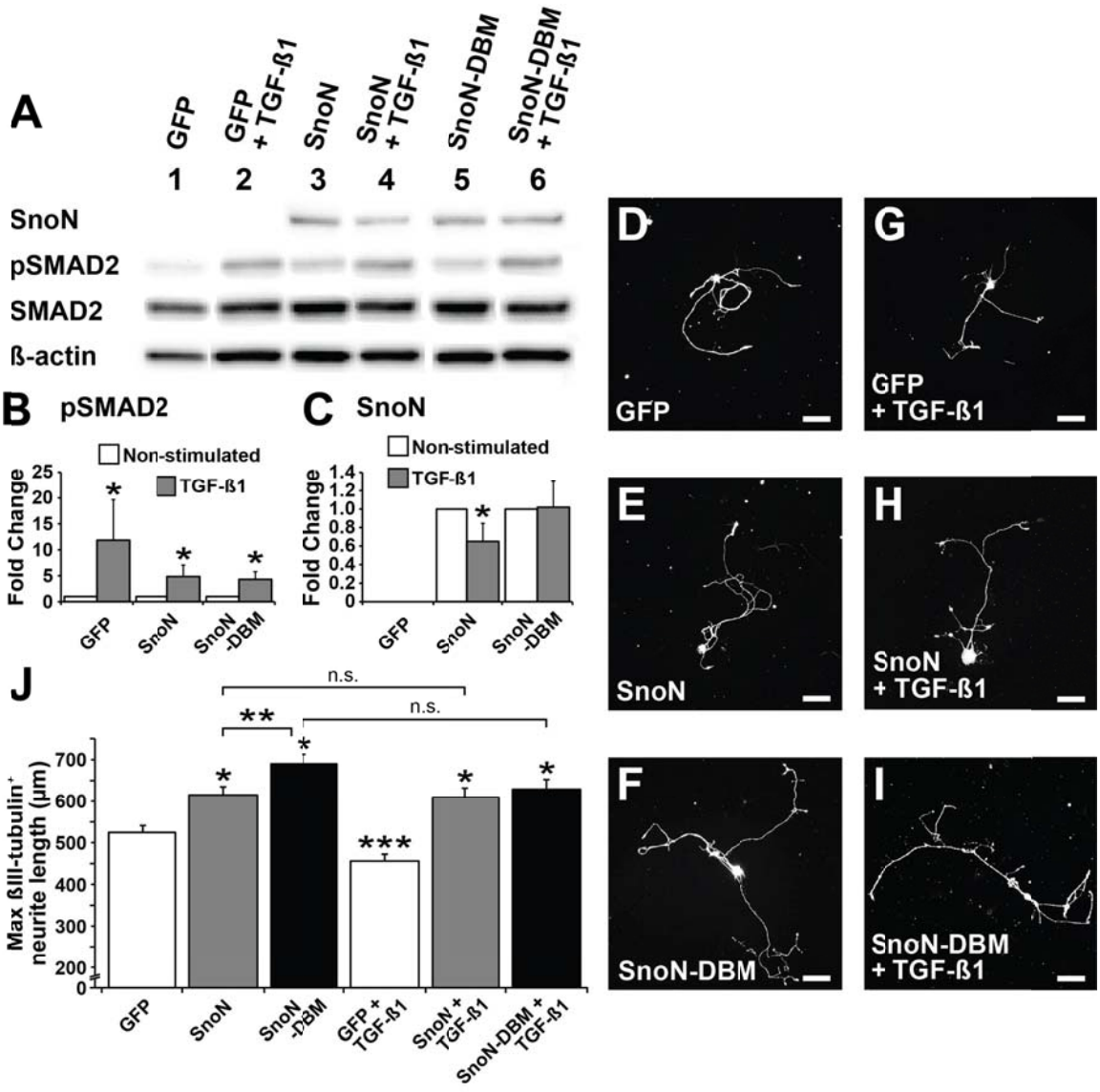


Figure 2.2: Sciatic Nerve Conditioning Lesion Reduces Inhibition by TGF-β1

Adult DRG neurons were peripherally conditioned 7 days prior to culture and addition of TGF-ß1. (**A**) Doses of 10 and 50 ng/ml TGF-ß1 were no longer inhibitory; only the highest dose of TGF-ß1 was partially inhibitory to measures of maximim neurite length (ANOVA $p<0.05$; *, post hoc Fisher's $p<0.05$) and (**B**) total neurite length (ANOVA $p<0.05$; *, post hoc Fisher's $p<0.05$). (**C**) Once again, TGF-ß1 did not affect the number of neurites emerging from the soma.

Figure 2.3: TGF-β and SnoN

(**A**) Western blot from adult DRG neurons expressing SnoN, SnoN-D-box mutation, or GFP after addition of 50 ng/mL TGF-β1 for 45 min. TGFß-1 addition leads to phosphorylation of SMAD2 (lane 2). Overexpression of SnoN or SnoN-D-box mutation leads to elevated levels of SnoN in DRG neurons (lanes 3 and 5), even following addition of TGFß-1 (lanes 4 and 6). SnoN-D-box mutation-expressing neurons are more resistant to reductions in SnoN levels following addition of TGFß-1 (lane 6) than neurons overexpression wild-type SnoN (lane 4). (**B**) Quantitation of blots in panel A shows SMAD2 phosphorylation after TGFß-1 addition, and (**C**) elevation of SnoN levels following neuronal transduction with SnoN or SnoN-D-box mutation. (ANOVA $p<0.05$; *, post hoc Fisher's $p<0.05$). (**D**) Neurite outgrowth from adult DRG neurons in vitro is (**E**) reduced following addition of TGFß-1. (**F**) Overexpression of SnoN increases neurite length, and (**G**) counters inhibitory effects of TGFß-1. (**H**) Overexpression of SnoN-D-box mutation further amplifies neurite growth, and (**I**) more extensively counters inhibitory effects of TGF-ß1. (**J**) Quantification confirms effects of SnoN on neurite growth and overcoming of TGFß-1-mediation inhibition. (ANOVA $p<0.001$; post hoc Fisher's *$p<0.05$, **$p<0.01$, ***$p<0.005$).


A
GFP
GFP + TGF-ß1
SnoN
SnoN + TGF-ß1
SnoN-DBM
SnoN-DBM + TGF-ß1
1
2
3
4
5
6
SnoN
pSMAD2
SMAD2
ß-actin
B pSMAD2
Non-stimulated
TGF-ß1
Fold Change
GFP
SnoN
SnoN -DBM
C SnoN
Non-stimulated
TGF-ß1
Fold Change
GFP
SnoN
SnoN -DBM
D
GFP
G
GFP + TGF-ß1
E
SnoN
H
SnoN + TGF-ß1
F
SnoN-DBM
I
SnoN-DBM + TGF-ß1
J
n.s.
**
n.s.
Max ßIII-tubulin+ neurite length (µm)
GFP
SnoN
SnoN -DBM
GFP + TGF-ß1
SnoN + TGF-ß1
SnoN-DBM + TGF-ß1

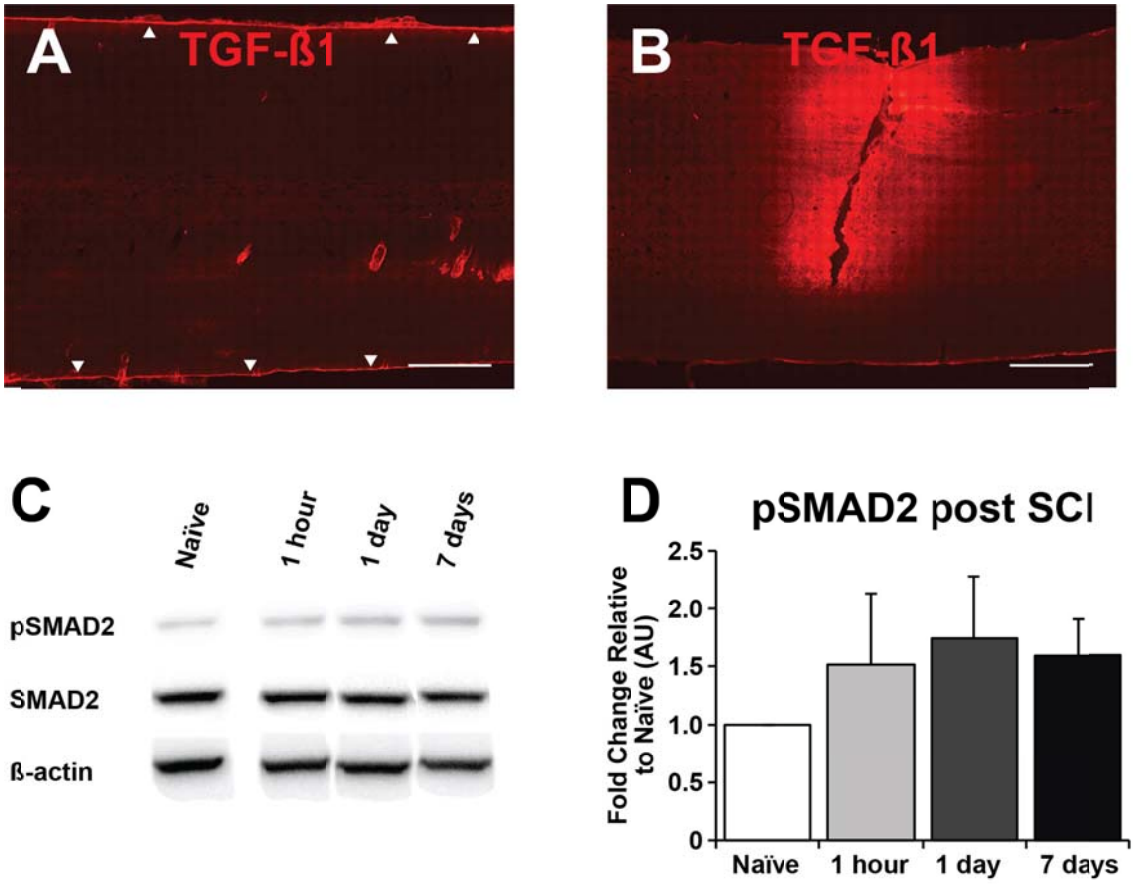


Figure 2.4: Injury Activates TGF-β1 Signaling *In Vivo*

(**A**) There is low basal expression of TGF-ß1 in the intact spinal cord with limited signal in the meninges (arrows). (**B**) One hour following C3 dorsal spinal cord transection, there is a marked upregulation of TGFß-1 immunoreactivity in the peri-lesioned region (arrows). (**C**) Western blot of lysates collected from L4 and L5 DRGs of animals that underwent C3 dorsal column lesions (C3) 1 hour, 1 day or 7 days earlier. Levels of phosphor-SMAD2 begin to increase one hour post-injury and remain elevated 1 day and 7 days later. (**D**) Quantitation of Western blot indicates approximate 2-fold elevation in phosphor-SMAD2 levels in DRG neurons after C3 spinal cord injury.

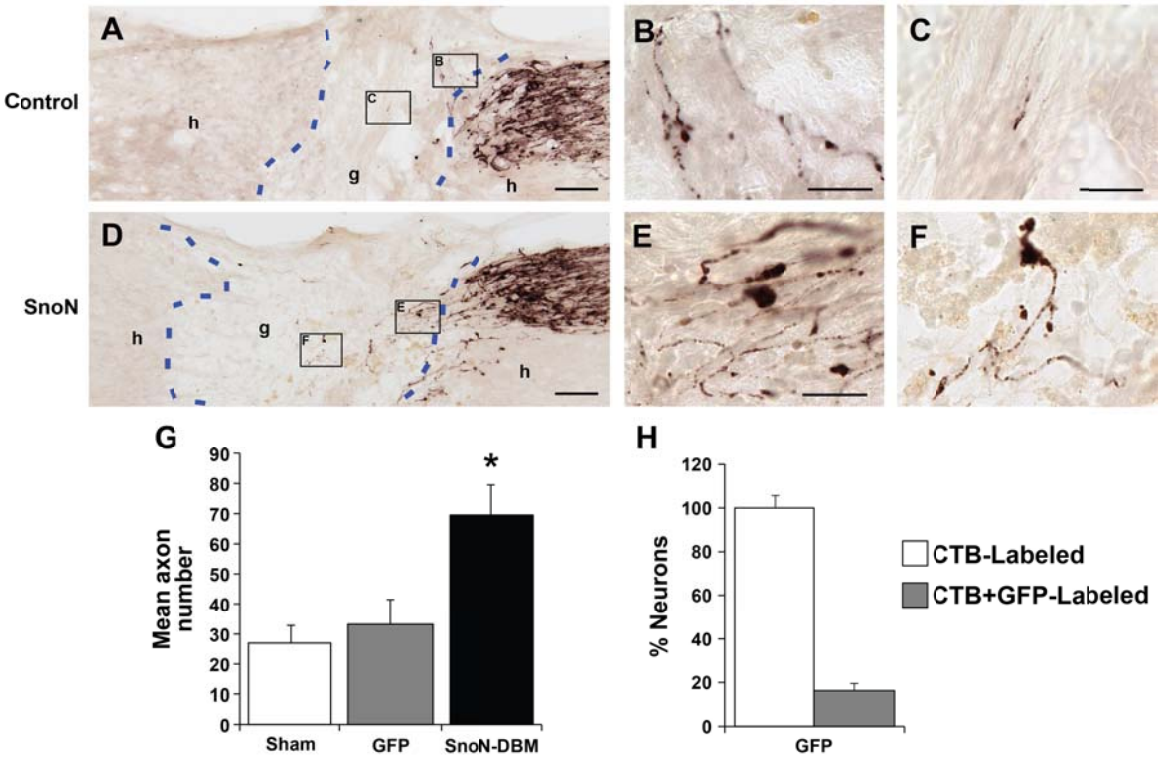
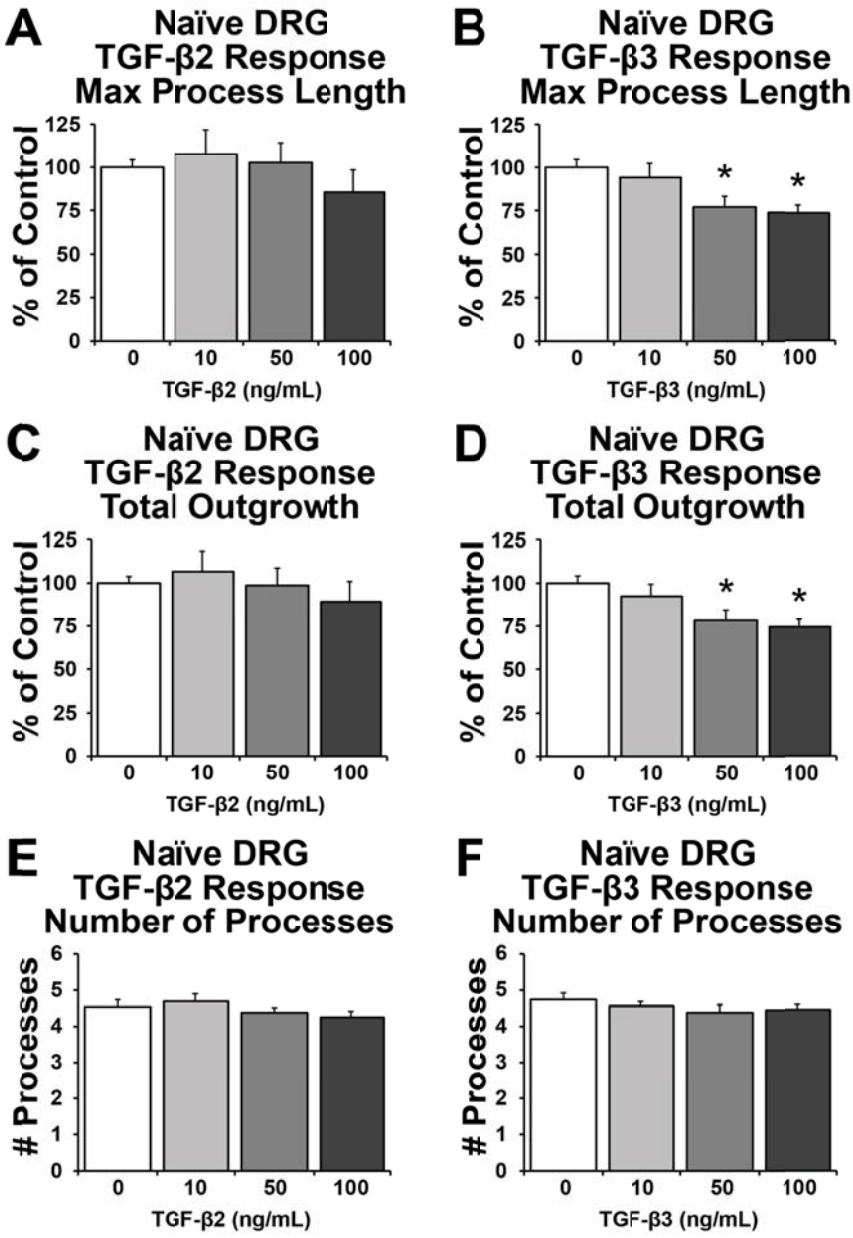


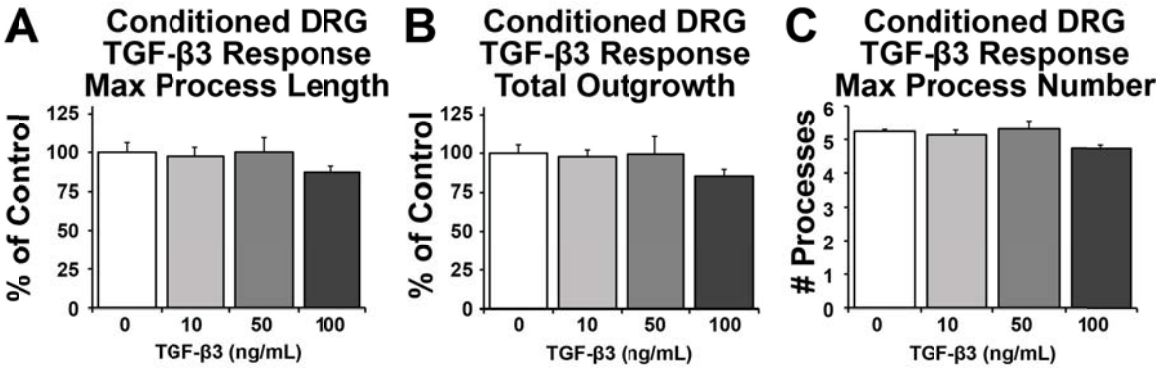
Figure 2.5: SnoN-DBM Overexpression is Sufficient to Facilitate Somatosensory Axon Regeneration *in vivo*

(**A**) Animals treated with only AAV6-GFP and naïve MSC grafts demonstrated occasional penetrations of CTB-labeled sensory axons into the graft. g, graft; h, host. Blue lines indicate host/graft interface. Scale bar: 100 μm. (**B**) Higher magnification of (A) showing the occasional axon in the graft of control GFP animals. (**C**) Higher magnification of (A) showing the axonal entry zone near the host/graft interface. (**D**) Treatment with AAV6-SnoN-DBM significantly enhanced CTB-labeled axon penetration into the graft. (**E** and **G**) Higher magnification images of CTB-labeled axons within the cell graft and complex arborizations. (**F**) Higher magnification image of the axonal entry zone from (D). (**G**) Quantification of CTB-labeled axons that extended to at least the midline of the graft. Animals that received injections of AAV6-SnoN-DBM exhibit a significantly greater number of axons within the graft compared to sham and GFP control groups (ANOVA $p<0.01$; **, post hoc Fisher's $p<0.05$). (**H**) Quantification of DRG cell bodies labeled with CTB or CTB and GFP in animals that received injections of AAV6-GFP or AAV6-SnoN-DBM. Scale bar in (A) and (D), 100 μm; in (B), (C), (E), (F), and (G), 25 μm.

Supplemental Figure 2.1: Select Alternative TGF-β Isoforms also Inhibit Outgrowth of Adult DRG Neurons

Adult DRG neurons isolated and cultured from female Fisher 344 rats plated on poly-L-lysine and laminin. (A-B) Treatment of adult DRGs with TGF-β3 (B) significantly decreased the length of the longest neurite at high concentrations (ANOVA $p<0.01$; *, post hoc Fisher's $p<0.05$). TGF-β2 (A) did not significantly affect longest neurite length. TGF-β3 (D) also decreased measurements of total outgrowth from adult DRG neurons (ANOVA $p<0.01$; *, post hoc Fisher's $p<0.05$) while TGF-β2 (C) did not significantly affect total outgrowth. Measurements of the number of processes were not significantly affected by TGF-β2 (E) or TGF-β3 (F).


A
Naïve DRG
TGF-β2 Response
Max Process Length
% of Control
125
100
75
50
25
0
0
10
50
100
TGF-β2 (ng/mL)
B
Naïve DRG
TGF-β3 Response
Max Process Length
% of Control
*
*
TGF-β3 (ng/mL)
C
Naïve DRG
TGF-β2 Response
Total Outgrowth
% of Control
TGF-β2 (ng/mL)
D
Naïve DRG
TGF-β3 Response
Total Outgrowth
% of Control
*
*
TGF-β3 (ng/mL)
E
Naïve DRG
TGF-β2 Response
Number of Processes
Processes
6
5
4
3
2
1
0
TGF-β2 (ng/mL)
F
Naïve DRG
TGF-β3 Response
Number of Processes
Processes
TGF-β3 (ng/mL)



Supplemental Figure 2. 2: Sciatic Nerve Conditioning Lesion Reduces Inhibition by TGF-β3

Adult DRG neurons were peripherally conditioned 7 days prior to culture and addition of TGF-ß1. (**A, B**) Doses of TGF-ß3 that were previously inhibitory no longer significantly reduced neurite outgrowth after conditioning, reflected in measures of both longest neurite (**A**) and total neurite length (**B**). (**C**) Once again, TGF-ß3 did not affect the number of neurites emerging from the soma.

This chapter, in its entirety, is currently being prepared for submission for publication of the material. Do JL, Bonni A, Tuszynski MH. The dissertation author was the primary investigator and first author of this paper.

CHAPTER 3

General Discussion

Recapitulating the developmental state when growth capacity is robust in injured adult neurons is a logical approach in order to promote regeneration of developed neurons following axotomy. Studies indicate that the bases for this developmental shift arises from changes in neuron-intrinsic mechanisms that alter cellular responses to stimuli and intracellular drivers of growth, neuron-extrinsic factors that promote or inhibit growth, and the convergence of intrinsic and extrinsic pathways through ligand-receptor mediated interactions (Maisonpierre *et al.*, 1990; Fitzgerald *et al.*, 1991). The studies described in this dissertation aim to address developmentally regulated pathways involving the convergence of intrinsic and extrinsic mechanisms that affect regeneration of lesioned central axons. This work demonstrated that while signaling pathways are necessary for axonal differentiation early in development, these same factors are sources of inhibition for regeneration in the mature nervous system and modulation of an intrinsic aspect of this pathway promotes adult regeneration. The following will discuss the major findings of this thesis work, potential implications for research in spinal cord injury, and future directions to pursue with respect to scientific inquiry and translational approaches to potential human therapies.

Modulation of Neuronal TGF-β Signaling Promotes Sensory Axon Regeneration Following Spinal Cord Injury

The TGF-β/activin subfamily of the TGF-β superfamily of morphogens is attributed with influencing a number of cellular processes. With regards to spinal cord injury, TGF-β is primarily associated with promoting scar formation and immune cell recruitment (Moon & Fawcett, 2001; Lagord *et al.*, 2002; Schachtrup *et al.*, 2010). However, TGF-β is also critical for axonal differentiation in early development (Yi *et al.*, 2010). Neuronal stimulation of this pathway is sufficient to induce development of multiple axons and promote axonal growth in developing neurons. However, in postnatal neurons, overexpression of SnoN, a canonical inhibitor of this signaling pathway and developmentally down-regulated transcription factor, promotes axon specific growth and is sufficient to overcome myelin induced growth inhibition (Stegmuller *et al.*, 2006; Stegmuller *et al.*, 2008). Though TGF-β demonstrates an essential role in neuronal growth early in development, a direct effect of TGF-β on adult neuronal growth has not been established and the potential role this essential pathway has on adult regeneration remains unexplored.

We found that adult neurons retain sensitivity to TGF-β ligands and express the canonical components of TGF-β signaling. Cultured sensory neurons respond to TGF-β stimulation by upregulating the phosphorylation of SMAD2. In the physiological state, sensory neurons injured by a spinal cord lesion at the distal processes in the central nervous system upregulate activation of TGF-β signaling related molecules within the cell soma. Furthermore, the activation of SMAD2 has the potential to promote the degradation of SnoN, a known promoter of axonal elongation. These findings indicate

that adult neurons do in fact have the capacity to directly respond to extracellular TGF-β ligand and that injury induces activation of this conserved pathway.

The observation that neurons directly respond to TGF-β logically led to the inquiry of whether TGF-β signaling retains influence on axonal growth as during development. We found that adult neuron growth was inhibited by TGF-β in a dose dependent manner. As a corollary, overexpression of SnoN, a canonical inhibitor of TGF-β signaling, increased adult neuron growth and, interestingly, overcame TGF-β induced inhibition.

These findings are in contrast to studies that have observed enhanced growth with TGF-β stimulation (Abe *et al.*, 1996; Yi *et al.*, 2010). However, in these instances, the effect was observed on embryonic or developing neurons as opposed to adult neurons. Mature neurons are genetically distinct from their embryonic counterparts, express a varied array of receptors and intracellular factors, and have well differentiated processes . Such differences may explain the observed reversal in TGF-β effects and follows a theme resonated in other signaling pathways and axon guidance models. MAG inhibits growth of adult neurons but stimulates growth of embryonic neurons (Turnley & Bartlett, 1998). Netrin-1 initially attracts growth cones in developing spinal cords through interaction with the DCC receptor but later becomes repulsive when those same growth cones express the UNC-5 receptor (Moore *et al.*, 2007).

While the direct neuronal inhibitory effect of TGF-β is novel, a more impressive finding was that the neuron-intrinsic manipulation of overexpressing SnoN promotes

growth in an in vivo spinal cord injury model. With SnoN overexpression, an increased number of fibers were found at the midpoint of the cell graft transplanted into the spinal cord lesion. These fibers represent truly regenerated fibers as they were clearly located within a cellular matrix devoid of host tissue, demonstrated nonlinear, branching morphologies, and could be seen penetrating the host-graft interface. Additionally, a lack of fibers rostral to the lesion indicates a complete lesion of the dorsal funiculus and reduces the probability that fibers observed within the cell graft were spared rather than regenerated.

As a proof of principle, this work successfully enhanced regeneration in the sensory system. This system is critical for conveying information regarding proprioception and fine touch but is not essential for motor function. As such, it would be informative to assess the effect of SnoN overexpression in other spinal circuits. While the raphespinal and rubrospinal systems do not considerably contribute to motor function in humans and non-human primates, these supraspinal circuits do contribute to motor function in rat and mouse models (Kennedy, 1990; Whishaw *et al.*, 1990; Liu *et al.*, 1999; Schucht *et al.*, 2002) and regeneration of these circuits may contribute a degree of functional recovery that is otherwise masked by the predominance of the corticospinal tract. Propriospinal circuits have also been found to contribute to spontaneous recovery following injury (Courtine *et al.*, 2008) and represent a potentially untapped substrate to improve behavioral outcomes.

These results indicate that modulation of TGF-β signaling is a viable approach to enhancing regeneration. To achieve functional regeneration, it is likely that a

combination of therapies will be necessary to achieve the anatomical changes such as increased axon growth, bridging of the lesion site, synapse onto the appropriate target, and myelination to bring about functional recovery. Combinatorial approaches have been shown to elicit a greater degree of regeneration than a single manipulation (Lu *et al.*, 2004; Houle *et al.*, 2006). By addressing multiple aspects that affect axonal growth that include extracellular inhibitory factors (McKeon *et al.*, 1991; McKerracher *et al.*, 1994; McKeon *et al.*, 1995; Wang *et al.*, 2002), loss of trophic support (Grill *et al.*, 1997; Tuszynski *et al.*, 2003; Brock *et al.*, 2010), and intracellular mechanisms (Gao *et al.*, 2004; Park *et al.*, 2008; Zou *et al.*, 2009; Liu *et al.*, 2010), the goal to achieve consistent functional regeneration may finally be realized.

Combinatorial approaches, even single factor approaches, however, are not without inherent risks. A number of potential candidates that promote regeneration, including SnoN, are not only related to development, but have also demonstrated a propensity to act as tumor suppressors and are critical for regulating cell cycle (Lamouille & Derynck, 2009; Lee & Muller, 2010; Pot *et al.*, 2010; Carracedo *et al.*, 2011). Thus, interventions that aim to manipulate multiple factors carry the potential to induce normal cells into a cancerous state. Thus, the development of strategies to enable tight regulation of these genetic therapies is essential to prevent exacerbation of an already precarious state. Fortunately, a number of strategies in development have the potential to control transgene expression and limit expression when it is no longer required (Gossen & Bujard, 1992; Gossen *et al.*, 1995; Alfa *et al.*, 2009).

Transgenic therapies represent novel advancements in scientific research and is a testament to the ingenuity of the scientist developing these tools; however, their application carries with it benefits, risks, and practical issues. While genes delivery with viral vectors allows for controlled, site-specific transgene expression, this benefit must but tempered with the reality that this application requires surgical intervention that may potentially cause greater damage to functionally beneficial spared tissue and introduces a substance recognized by the body as foreign. Further, such intervention presents a practical concern in that facilities would require specialization to deliver these agents safely and reliably.

To circumvent such risks and modulate TGF-β signaling in a more practical manner, a pharmacological approach could be developed. The significance of TGF-β in cancer biology has resulted in the synthesis of a number of compounds with the potential to inhibit TGF-β signaling (Waghabi *et al.*, 2007; Ichida *et al.*, 2009; Mohammad *et al.*, 2011). To rapidly screen such compounds for effects in neuronal populations, we propose developing an immortalizated neuronal cell line that responds to TGF-β regulation with changes in an easily detectable reporter. Viral introduction of a GFP or luciferase regulated by TGF-β responsive elements (Riccio *et al.*, 1992; Massague, 1998) into an already existing neuronal cell lines (Wood *et al.*, 1990; Chen *et al.*, 2007) could be a means to accomplish this goal. This approach would yield information regarding compound efficiency in regulating neuronal TGF-β signaling and guide further studies to examine effects on axon growth and translational applicability.

Conclusion

Functional recovery following SCI necessitates axon growth beyond the capacity observed in the endogenous state. The present work demonstrates that modulation of the previously unappreciated, direct neuronal effects of TGF-β signaling enhances axon regeneration and may contribute to a comprehensive strategy to promote axon regeneration. Neuronal SnoN over-expression enhances the ability of axons to penetrate a cellular graft, thereby increasing the number of axons available to respond to treatments beyond the lesion and ultimately promote axon bridging and synapse formation. Thus, SnoN over-expression in conjunction with combinatorial approaches may be an important component of future SCI therapies.

References

Abe, K., Chu, P.J., Ishihara, A. & Saito, H. (1996) Transforming growth factor-beta 1 promotes re-elongation of injured axons of cultured rat hippocampal neurons. *Brain research*, **723**, 206-209.

Alfa, R.W., Tuszynski, M.H. & Blesch, A. (2009) A novel inducible tyrosine kinase receptor to regulate signal transduction and neurite outgrowth. *Journal of neuroscience research*, **87**, 2624-2631.

Brock, J.H., Rosenzweig, E.S., Blesch, A., Moseanko, R., Havton, L.A., Edgerton, V.R. & Tuszynski, M.H. (2010) Local and remote growth factor effects after primate spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 9728-9737.

Carracedo, A., Alimonti, A. & Pandolfi, P.P. (2011) PTEN level in tumor suppression: how much is too little? *Cancer Res*, **71**, 629-633.

Chen, W., Mi, R., Haughey, N., Oz, M. & Hoke, A. (2007) Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line. *Journal of the peripheral nervous system : JPNS*, **12**, 121-130.

Courtine, G., Song, B., Roy, R.R., Zhong, H., Herrmann, J.E., Ao, Y., Qi, J., Edgerton, V.R. & Sofroniew, M.V. (2008) Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. *Nat Med*, **14**, 69-74.

Fitzgerald, M., Reynolds, M.L. & Benowitz, L.I. (1991) GAP-43 expression in the developing rat lumbar spinal cord. *Neuroscience*, **41**, 187-199.

Gao, Y., Deng, K., Hou, J., Bryson, J.B., Barco, A., Nikulina, E., Spencer, T., Mellado, W., Kandel, E.R. & Filbin, M.T. (2004) Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. *Neuron*, **44**, 609-621.

Gossen, M. & Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. & Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766-1769.

Grill, R., Murai, K., Blesch, A., Gage, F.H. & Tuszynski, M.H. (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **17**, 5560-5572.

Houle, J.D., Tom, V.J., Mayes, D., Wagoner, G., Phillips, N. & Silver, J. (2006) Combining an autologous peripheral nervous system "bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **26**, 7405-7415.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K., Huangfu, D., Akutsu, H., Liu, D.R., Rubin, L.L. & Eggan, K. (2009) A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. *Cell Stem Cell*, **5**, 491-503.

Kennedy, P.R. (1990) Corticospinal, rubrospinal and rubro-olivary projections: a unifying hypothesis. *Trends Neurosci*, **13**, 474-479.

Lagord, C., Berry, M. & Logan, A. (2002) Expression of TGFbeta2 but not TGFbeta1 correlates with the deposition of scar tissue in the lesioned spinal cord. *Mol Cell Neurosci*, **20**, 69-92.

Lamouille, S. & Derynck, R. (2009) Oncogene and tumour suppressor: the two faces of SnoN. *The EMBO journal*, **28**, 3459-3460.

Lee, E.Y. & Muller, W.J. (2010) Oncogenes and tumor suppressor genes. *Cold Spring Harb Perspect Biol*, **2**, a003236.

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B. & He, Z. (2010) PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature neuroscience*, **13**, 1075-1081.

Liu, Y., Kim, D., Himes, B.T., Chow, S.Y., Schallert, T., Murray, M., Tessler, A. & Fischer, I. (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **19**, 4370-4387.

Lu, P., Yang, H., Jones, L.L., Filbin, M.T. & Tuszynski, M.H. (2004) Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. *J Neurosci*, **24**, 6402-6409.

Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M. & Yancopoulos, G.D. (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron*, **5**, 501-509.

Massague, J. (1998) TGF-beta signal transduction. *Annu Rev Biochem*, **67**, 753-791.

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

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

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J. & Braun, P.E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron*, **13**, 805-811.

Mohammad, K.S., Javelaud, D., Fournier, P.G., Niewolna, M., McKenna, C.R., Peng, X.H., Duong, V., Dunn, L.K., Mauviel, A. & Guise, T.A. (2011) TGF-beta-RI

kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. *Cancer Res*, **71**, 175-184.

Moon, L.D. & Fawcett, J.W. (2001) Reduction in CNS scar formation without concomitant increase in axon regeneration following treatment of adult rat brain with a combination of antibodies to TGFbeta1 and beta2. *Eur J Neurosci*, **14**, 1667-1677.

Moore, S.W., Tessier-Lavigne, M. & Kennedy, T.E. (2007) Netrins and their receptors. *Advances in experimental medicine and biology*, **621**, 17-31.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M. & He, Z. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*, **322**, 963-966.

Pot, I., Ikeuchi, Y., Bonni, A. & Bonni, S. (2010) SnoN: bridging neurobiology and cancer biology. *Curr Mol Med*, **10**, 667-673.

Riccio, A., Pedone, P.V., Lund, L.R., Olesen, T., Olsen, H.S. & Andreasen, P.A. (1992) Transforming growth factor beta 1-responsive element: closely associated binding sites for USF and CCAAT-binding transcription factor-nuclear factor I in the type 1 plasminogen activator inhibitor gene. *Mol Cell Biol*, **12**, 1846-1855.

Schachtrup, C., Ryu, J.K., Helmrick, M.J., Vagena, E., Galanakis, D.K., Degen, J.L., Margolis, R.U. & Akassoglou, K. (2010) Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J Neurosci*, **30**, 5843-5854.

Schucht, P., Raineteau, O., Schwab, M.E. & Fouad, K. (2002) Anatomical correlates of locomotor recovery following dorsal and ventral lesions of the rat spinal cord. *Experimental neurology*, **176**, 143-153.

Stegmuller, J., Huynh, M.A., Yuan, Z., Konishi, Y. & Bonni, A. (2008) TGFbeta-Smad2 signaling regulates the Cdh1-APC/SnoN pathway of axonal morphogenesis. *J Neurosci*, **28**, 1961-1969.

Stegmuller, J., Konishi, Y., Huynh, M.A., Yuan, Z., Dibacco, S. & Bonni, A. (2006) Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron*, **50**, 389-400.

Turnley, A.M. & Bartlett, P.F. (1998) MAG and MOG enhance neurite outgrowth of embryonic mouse spinal cord neurons. *Neuroreport*, **9**, 1987-1990.

Tuszynski, M.H., Grill, R., Jones, L.L., Brant, A., Blesch, A., Low, K., Lacroix, S. & Lu, P. (2003) NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection. *Exp Neurol*, **181**, 47-56.

Waghabi, M.C., Keramidas, M., Calvet, C.M., Meuser, M., de Nazare, C.S.M., Mendonca-Lima, L., Araujo-Jorge, T.C., Feige, J.J. & Bailly, S. (2007) SB-431542, a transforming growth factor beta inhibitor, impairs Trypanosoma cruzi infection in cardiomyocytes and parasite cycle completion. *Antimicrob Agents Chemother*, **51**, 2905-2910.

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L. & He, Z. (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature*, **417**, 941-944.

Whishaw, I.Q., Tomie, J.A. & Ladowsky, R.L. (1990) Red nucleus lesions do not affect limb preference or use, but exacerbate the effects of motor cortex lesions on grasping in the rat. *Behav Brain Res*, **40**, 131-144.

Wood, J.N., Bevan, S.J., Coote, P.R., Dunn, P.M., Harmar, A., Hogan, P., Latchman, D.S., Morrison, C., Rougon, G., Theveniau, M. & et al. (1990) Novel cell lines display properties of nociceptive sensory neurons. *Proc Biol Sci*, **241**, 187-194.

Yi, J.J., Barnes, A.P., Hand, R., Polleux, F. & Ehlers, M.D. (2010) TGF-beta signaling specifies axons during brain development. *Cell*, **142**, 144-157.

Zou, H., Ho, C., Wong, K. & Tessier-Lavigne, M. (2009) Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci*, **29**, 7116-7123.