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Sox9 conditional knockdown reduces chondroitin sulphate proteoglycan expression, increases neuroplasticity, and improves motor function in a mouse model of spinal cord injury

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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SOX9 CONDITIONAL KNOCKDOWN REDUCES CHONDROITIN SULFATE PROTEOGLYCAN EXPRESSION, INCREASES NEUROPLASTICITY, AND IMPROVES MOTOR FUNCTION IN A MOUSE MODEL OF SPINAL CORD INJURY

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by

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Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

This thesis investigates the effect of Sox9 knockdown on anti-regenerative scar gene expression, neuroplasticity, and hind limb functional recovery following mouse spinal cord injury. We hypothesized that Sox9 knockdown would reduce expression of anti-regenerative chondroitin sulfate proteoglycans both at the lesion site and at sites distant to the injury, thus providing an avenue for increased neuroplasticity and locomotor recovery after spinal cord injury. The first chapter provides a general introduction to the biological problem of spinal cord injury. The development of the glial scar and expression of the anti-regenerative chondroitin sulfate proteoglycan (CSPG) extracellular matrix is introduced, and Sox9 is identified as a transcription factor that may control expression of these anti-regenerative genes. The second chapter is a manuscript that describes the molecular changes and improved locomotor function seen when Sox9 knockdown is carried out just prior to spinal cord injury. The third chapter is more clinically relevant as it is a manuscript detailing the effects of Sox9 knockdown after spinal cord injury on the recovery of hind limb motor function. The fourth chapter is a manuscript investigating the neuro-anatomical mechanism of the improved functional recovery seen in Sox9 knockdown mice after spinal cord injury. The fifth chapter reflects on the findings presented herein, and suggests possible future plans of study. This dissertation demonstrates that inhibition of Sox9 leads to reduced CSPG expression, improved hind limb function, and increased total locomotion. It further provides compelling evidence that increased neuroplasticity as evidenced by increased reactive sprouting and increased expression of the presynaptic markers synaptophysin and VGLUT1 caudal to the injury site underlies the improved neurological recovery observed in spinal cord injured Sox9 conditional knockdown mice.

Key words: spinal cord injury, Sox9, chondroitin sulfate proteoglycans, neuroplasticity, reactive sprouting
Co-authorship

Chapter 2 of this dissertation was co-authored for publication by myself, Magda Dragan, Dr. Andreas Schedl, and Dr. Arthur Brown. Magda Dragan was instrumental in developing astrocyte cell culture techniques. Chapter 3 of this dissertation has been submitted for publication co-authored by myself, Eli York, and Dr. Arthur Brown. Eli York assisted in the day to day care of the mice used for the delayed knockdown experiment, the evaluation of their locomotor activity, and the spinal tissue cryosectioning as well as GFAP and CSPG immunostaining of sectioned spinal cords. Chapter 4 of this dissertation has been prepared for publication co-authored by myself, Dr. Todd Hryciw, Dr. Kathy Xu, Dr. Nicole Geremia, and Dr. Arthur Brown. Dr. Todd Hryciw performed the BDA injections into the mouse primary motor cortex. Dr. Kathy Xu helped to develop the BDA and fluorogold quantification techniques. Dr. Nicole Geremia helped to develop the fluorogold administration technique. In each of these studies, I planned and executed the experiments and completed the analysis of results with the aforementioned assistance. Dr. Andreas Schedl contributed the Sox9<sup>flox/flox</sup> mice for our usage. Dr. Arthur Brown conceptualized much of the experimental design, provided appropriate guidance, assisted with technical problems, and edited manuscripts. Dr. Arthur Brown also secured the funding and provided the facilities required to complete this work.
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Thank you to my supervisor and mentor Dr. Arthur Brown and to the members of my advisory committee, Dr. Vania Prado, Dr. Nagalingam Rajakumar, and Dr. Lique Coolen for their guidance and assistance. I would also like to thank Dr. Greg Dekaban for all of his assistance over the years, and Dr. Lynne Weaver for help with manuscript preparation.

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Dedication

This work is dedicated to anyone with a spinal cord injury
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List of Abbreviations

AANS/CNS – American Association of Neurological Surgeons/Congress of Neurological Surgeons

ANOVA – analysis of variance

BBB – blood brain barrier

BMS – Basso Mouse Scale

BDNF – brain derived neurotrophic factor

C4ST – Chondroitin-4 sulfotransferase

CNS – central nervous system

CPG – central pattern generator

CSPG – chondroitin sulfate proteoglycans

DRG – dorsal root ganglion

ECM – extracellular matrix

GAG – glycosaminoglycans

GalNAc – N-acetylgalactosamine

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

GlcA – glucuronic acid
HMG – high-mobility-group

HRP – horse radish peroxidase

HSPG – heparin sulfate proteoglycans

IL – interleukin

LAR - leukocyte common antigen-related phosphatase receptor

MAG – myelin associated glycoprotein

MBP – myelin basic protein

MP – methylprednisolone

NASCIS – National Acute Spinal Cord Injury Study

PDGF – platelet derived growth factor

PGC-1alpha – Peroxisome proliferator-activated receptor gamma co-activator 1alpha

PNN – perineuronal net

PNS – peripheral nervous system

RPTPσ – receptor protein tyrosine phosphatase sigma

RT-PCR – real time PCR

SCI – spinal cord injury

SOX – Sry-type HMG box transcription factor
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TGF – transforming growth factor

VGAT – vesicular GABA transporter

VGLUT – vesicular glutamate transporter

XT – xylosyltransferase

YFP – yellow fluorescent protein
Chapter 1: Introduction

1.0 Spinal cord injury

The spinal cord serves as the communication relay between the brain and the periphery. It conveys messages from the brain to control movement, breathing, and other bodily functions, and messages from sensory organs to the brain to allow us to interpret and act on those sensations. These messages are transmitted by long extensions of neurons called axons. The spinal cord is surrounded by vertebrae that protect it. If these bones are damaged, the spinal cord and the axons therein may be injured and vital information that needs to be transmitted to, or from, the brain does not reach its destination.

Spinal cord injury (SCI) is a devastating event resulting in immediate life-altering consequences for not only the affected individual but their family and friends. SCI can result in motor, sensory, and autonomic dysfunctions [1]. Depending on the severity of injury, SCI may leave patients with lifetime disability [1]. Although paralysis is the symptom most people associate with SCI, those who suffer a SCI may also develop numerous other debilitating symptoms including chronic pain [2], muscle spasticity [3], poor control of bladder and bowel function [4], and decreased sexual function [5, 6]. SCI resulting in permanent paralysis or significant neurological deficit occurs with an annual incidence between 25 and 93 cases per million North Americans [7]; in Canada this translates to over 1,000 new cases a year. SCI often has a profound socio-economic impact on people as they may be forced to leave their jobs, require ongoing medical care for the duration of their lives, and incur massive patient health care
costs [8]. This socio-economic burden is exacerbated by the fact that these injuries are most common in young adults [7].

SCI occurs in a two-stage process. The initial stage is known as the primary SCI which occurs at the time of injury due to physical trauma to the spinal cord [9]. Examples of primary injury include hyperextension of the cord, destruction due to sheering or twisting of the cord, vertebral fracture and contact with the side of the cord, and laceration due to a direct cut of the cord by sharp object, or projectile [10]. Most often these injuries also result in reduction of blood flow to the spinal cord resulting in ischemia [11] that contributes significantly to the destruction of the grey matter which requires considerable oxygen supply [10]. Damage to the spinal cord is not limited to this initial physical injury. A secondary phase of SCI begins in the hours following the initial trauma to the cord. This secondary SCI is characterized by the body’s activation of inter-related processes that lead to an expansion of the lesion and contribute to neuronal loss and increased functional defect [12]. Such secondary processes contributing to SCI include ischemia-reperfusion [13], edema [14], excitotoxicity [15], oxidative damage [16], inflammation [17], and glial scarring [18].

The debilitating neurological damage that results as a consequence of SCI has long been considered to be irreversible. Currently, there are no effective pharmacological treatments for SCI [19]. Thus, improved therapeutic strategies for the treatment of SCI need to be developed. A beneficial treatment for SCI will need to limit neuronal damage and subsequent functional degeneration, or promote axonal regeneration culminating in significant functional recovery. The project described herein focuses on the promotion of axonal regeneration post-SCI.
1.1 Nervous system plasticity

Embryonic central nervous system (CNS) neurons display significant plasticity, however in the adult CNS this intrinsic ability to grow becomes suppressed to maintain proper synaptic organization [20]. Unlike embryonic CNS neurons, mature CNS neurons display minimal neurite outgrowth, do not respond readily to neurotrophic stimuli, are significantly inhibited by myelin-associated inhibitors, and form few growth cones [21, 22]. Much like CNS neurons, peripheral neurons undergo similar reduction in their growth properties over time, but their intrinsic growth capabilities can be reactivated by peripheral nerve injury that may result in increased expression of pro-regeneration transcription factors and growth associated proteins thus allowing for considerable peripheral nerve regeneration [20, 23, 24]. This type of reactivation of pro-regenerative transcription factors is not seen in response to CNS injuries, and thus CNS neurons do not regenerate to the same extent as peripheral nervous system (PNS) neurons post-injury. However, this is not the sole reason why CNS neurons do not regenerate to the same extent as PNS neurons. The PNS contains Schwann cells, and axons from both PNS and CNS neurons grow well in a Schwann cell rich environment [25-27]. The CNS, however, contains astrocytes and oligodendrocytes rather than Schwann cells, and culture experiments have shown that both PNS and CNS axons grow poorly across astrocyte and oligodendrocyte rich environments [28-30]. Following CNS damage, axonal and glial debris remains for a significant period of time, often months, and has been suggested to act as a source of axonal growth inhibition. In comparison, post-injury debris is cleared much faster in the PNS [31, 32]. Perhaps the most significant impediment to axonal growth in the CNS is the response of cells located at the local site of injury. Unlike in the periphery [33], the adult CNS experiences the activation of astrocytes, and development of a subsequent astroglial scar. CNS trauma often results in a
disruption of the blood–brain barrier, which allows neutrophils and macrophage to extravasate from the blood to assist endogenous microglia with antigen recognition and phagocytic functions. Fibroblasts from the meninges converge with the inflammatory cells to the injury site and release various cytokines and chemokines [34, 35]. These small molecules activate reactive astrocytes to induce further gliosis and glial scar formation. This glial scar consists of a number of growth inhibiting molecules that contribute to the failure of axon regeneration [18, 36-42]. Many of the molecules (semaphorins, ephrins, netrins and slit) that are expressed after injury in the glial scar induce axonal growth cone collapse. Studies investigating the inhibition of these molecules have demonstrated some promise for the functional recovery after CNS injuries including SCI [43, 44]. There are also many myelin-derived inhibitors such as Nogo, myelin-associated glycoprotein, repulsive guidance molecule, and oligodendrocyte myelin glycoprotein each of which inhibit neurite outgrowth in vitro [45]. However, their contribution in vivo is still under investigation as Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein triple knockout mice do not display improved recovery post-SCI [46, 47]. In 1990 Silver et al. found that a major component of the glial scar, chondroitin sulfate proteoglycans (CSPGs) that are up-regulated post-neurotrauma [48], prevent axonal growth as cultured axons from the E9 chick dorsal ganglia would not grow across a strip of CSPGs coated on nitrocellulose [49]. CSPG digestion abolished this inhibition and allowed the chick axons to grow across the plate. Thus, CSPGs became a focus for the investigation of axonal growth inhibitory molecules.
1.2 Chondroitin Sulfate Proteoglycans (CSPGs)

CSPGs are a class of extracellular matrix macromolecules that share a common structure consisting of a central core protein with a number of chondroitin sulfate side chains attached [50]. More than 10 enzymes participate in the complex synthesis of a completed CSPG. First, the CSPG core protein is produced. Next a tetrasaccharide linker is synthesized. The rate limiting step in CSPG synthesis is the attachment of this tetrasaccharide linker to the CSPG core protein by way of a serine-xyline interaction catalyzed by the enzyme xylosyltransferase (XT) isoforms I and II (XT-I, XT-II) [51]. Chondroitin sulfate N-acetylgalactosaminyltransferase adds N-acetylgalactosamine (GalNAc) to the tetrasaccharide linker [52], and chondroitin sulfate synthase, as well as chondroitin polymerizing factor, add glucuronic acid (GlcA) to the available N-acetylgalactosamine [53, 54]. This polymerization process repeats with the addition of subsequent (GalNAc-GlcA) disaccharides one after another to form a long carbohydrate chain. Finally, these side chains are sulfated by chondroitin 4-sulfotransferase (C4ST) [55] to make a complete CSPG.

There are many different types of CSPGs, each defined by their core protein. There are large aggregating proteoglycans including aggrecan [56] and versican [57] localized in most smooth muscle tissues and in fibrous and elastic cartilage as well as the CNS, and CNS-specific proteoglycans neurocan [58], brevican[59], NG2 [60], and phosphacan [61]. These CSPGs exist abundantly within the CNS and interact with a variety of other ECM components including laminin, fibronectin, tenascin, hyaluronic acid and several collagens by way of interaction through their core protein, or sugar side chain [57, 62-65].
CSPG expression is tightly controlled in the embryonic brain, with expression restricted during development to highly specialized and organized regions such as neural crest pathways and the spinal cord where they act as axon guidance molecules [66, 67]. The removal of these CSPGs results in abnormally formed axonal pathways [68]. In the adult CNS, the normal expression patterns of CSPGs become more diffuse. Neurocan (produced by astrocytes and oligodendrocyte precursor cells) is generally found in the white matter of the adult CNS [69]. NG2 is distributed throughout the adult brain existing on oligodendrocyte precursor cells [70], on blood vessels and meningeal cells [50]; and is often cleaved and secreted into the ECM [71]. Phosphacan is present throughout the CNS and at particularly high levels in the cerebellum [72]. Versican is produced by oligodendrocyte precursor cells and is found in high concentration in the white matter, [73]. Brevican is distributed throughout the CNS [74] and is produced predominantly by astrocytes [75]. CSPGs also amass in extracellular matrix structures which surround synapses on the neuronal surface known as perineuronal networks (PNNs) [76]. This CSPG recruitment into PNN structures coincides with the end of the critical period of plasticity which occurs in the transition to adulthood [76]. These CSPGs exist normally within the adult CNS [77], but following SCI, their expression levels markedly increase [37, 78]. CSPGs become up-regulated to play a beneficial role post-injury as they surround the lesion site and disrupted blood-brain barrier to seal off the damaged tissue, limit the secondary inflammatory response, and prevent increased cavitation [79]. However, as previously mentioned, CSPGs both in the lesion site and in PNNs also pose a significant impediment to regeneration post neurotrauma.
1.3 Regenerative failure post-SCI – CSPGs in the glial scar

Following SCI, axonal regeneration in the adult CNS is poor. However, axons do have an intrinsic ability to grow in both the CNS tissue and degenerating white matter. Davies et al. [42] demonstrated that adult dorsal root ganglion (DRG) sensory neurons transplanted into the white matter tracts of adult rats could grow in the CNS. If the transplantation procedure caused minimal damage to the local transplantation site, considerable numbers of regenerating adult axons rapidly extended through the white matter and eventually invaded the grey matter. If the surgical procedure caused considerable damage to the local area, failure in long range regeneration was noted. Increased CSPG expression was seen within the extracellular matrix at the transplant interface in those animals that displayed abortive regeneration, and minimal CSPG expression was noted in animals which displayed successfully regenerating transplants [42]. In a follow up study Davies et al. [36] transplanted DRG sensory neurons into degenerating white matter of the adult rat spinal cord several millimeters rostral to a severe lesion of the dorsal columns. DRG axonal growth away from the transplantation site, in both the rostral and caudal directions, was noted. Upon reaching the lesion site the rapidly extending axons stopped their growth, experienced growth cone collapse, and became dystrophic. High concentrations of CSPGs were found at the site of cessation of growth in the reactive glial matrix. These results suggested that the major impediment to axonal regeneration in the adult CNS was the molecular barrier found in the glial scar that forms at the lesion site, and that inhibition of axon growth correlates directly with elevated CSPG expression.

In vitro studies have shown that explanted glial tissue expressing CSPGs do not permit neurite extension [37]. In cultures of primary astrocytes, neurites avoid patches of cells
expressing CSPGs [80]. Specific CSPGs have also been shown to inhibit neurite outgrowth including: aggrecan [81], neurocan [82], phosphocan [83], brevican [75], versican [84], and NG2 [85]. Neurocan in particular has been identified as a major impediment to recovery post-SCI [86]. Importantly, following mid-thoracic SCI neurocan expression is increased throughout the lesion site both in the cervical dorsal columns and in the lumbar ventral horn much longer than other CSPGs [86]. The long lasting increase of neurocan in gray matter regions at distal levels of the spinal cord may contribute to the restriction of plasticity in the chronic phase after SCI.

The mechanism behind CSPG inhibition of axonal growth is believed to be due to both the physical and molecular barrier that CSPGs impose at the glial scar. The negatively charged sulfates present on the sugar side chains are repellent to axon fibers [87] and CSPGs sterically inhibit access to substrate adhesion molecules [88]. Recently, the transmembrane receptor protein tyrosine phosphatase sigma (RPTPσ) has been identified as an axonal high affinity receptor for CNS CSPGs [89]. Following interaction with CSPGs, RPTPσ signals growth cone collapse and cessation of axonal growth [90-92]. In vitro work demonstrated that cerebellar granule neurons from RPTPσ knockdown mice display reduced sensitivity to CSPG growth inhibition [93]. In vivo, corticospinal tract axons were found to regenerate through the injury site and extend for long distances after a dorsal hemisection or contusion injury of the thoracic spinal cord in RPTPσ deficient mice [93]. Transmembrane leukocyte common antigen-related phosphatase receptor (LAR), another member of the RPTP family, has also been identified as a receptor for CSPGs [94]. Functional blockade of LAR reversed neurite growth inhibition induced by CSPGs and induced significant descending axonal growth and locomotor functional recovery in mice with thoracic SCI [94].
CSPG up-regulation in the glial scar at the local injury site is certainly an obstacle to regeneration post-SCI; however, CSPG inhibition of axonal regeneration does not only occur at the glial scar. CSPGs also act as natural endogenous regulators of synaptic plasticity and experience-dependent neural plasticity as the principle component of the perineuronal network at sites distant to injury, and can thus restrict axonal plasticity throughout the CNS [95-97].

1.4 The perineuronal network

PNNs are composed of a highly condensed extracellular matrix that exists around the end feet of astrocytes surrounding the cell bodies and dendrites of CNS neurons [98]. PNNs were originally reported by Camillo Golgi in 1898 [99], and have since been confirmed to be produced by both neurons [100] and glia [101, 102]. PNNs are believed to play a number of functions in the adult brain; they maintain cellular positioning [103]; they modulate the chemotactic gradient of growth factors around neurons [98]; and they generate a polyanionic ion-buffering microenvironment to promote cell survival [104]. PNNs contain CSPGs, and tenascin-R [105], both non-permissive strata for axonal growth or attachment [106, 107]. This suggests that PNNs impede the formation of new synaptic contacts, and restrict axonal plasticity. In fact, PNNs play a crucial role in regulation of what is referred to as the critical period of synaptic plasticity in the brain.

As the juvenile brain develops, particular systems display critical periods of plasticity where the neuronal circuitry controlling these systems shows significantly increased sensitivity to experience [108-110]. Experience during these critical periods is believed to have a significant effect on how these neuronal systems interconnect; thus contributing to learning specific skills
and behaviors including interpretation of one’s senses and emotional processing [110, 111]. This critical period comes to an end with a dramatic up-regulation of PNNs around neuronal cell bodies and dendritic synapses bringing to a close this period of increased plasticity [98], finalizing the adaptations to the neuronal network acquired during the critical period [112-114]. The PNNs contain CSPGs that stabilize those synapses formed by critical period experience, preventing the formation of inappropriate synapses in the future by stunting axonal sprouting onto incorrect targets after appropriate connections have been made [97, 101, 115-117]. This mechanism appears to function as a method to retain proper understanding of sensation and optimized motor behaviors without need for continual maintenance, as constantly plastic synapses might leave individuals unable to use that circuitry optimally, and thus would be unable to interact optimally with their environment. Multiple studies have shown that reducing CSPG levels at the lesion site and/or in PNNs results in increased structural neuroplasticity [118-122]. Thus, therapies targeting a reduction in CSPGs both near and far from the lesion epicenter may present an interesting strategy for improved recovery post-SCI.

1.5 Anti-CSPG strategies show promise for the treatment of SCI

Thus far, three strategies have been devised to have broad effects on CSPGs and have been shown to improve axonal regeneration after SCI. The first strategy makes use of enzymatic digestion of the chondroitin sulfate side chains found on all CSPGs using the enzyme chondroitinase ABC. Chondroitinase treatment renders glia permissive to neurite outgrowth in vitro [118], and intrathecal chondroitinase treatment in the rat resulted in minor improvements in recovery and regeneration of ascending axonal tracts post-SCI [119], Axonal regeneration
following chondroitinase treatment of the injured spinal cord was modest. The lack of extensive improvement was attributed to the carbohydrate stubs left on the CSPG core proteins after chondroitinase treatment [123]. Another strategy was devised to block glycosylation of proteoglycan core proteins using an anti-XT-1 ribozyme administered after SCI, and resulted in improved axonal regeneration [124]. A third approach investigated mice carrying a gene knockdown for Chondroitin sulfate N-acetylgalactosaminyltransferase-1, a key enzyme in CSPG biosynthesis. N-acetylgalactosaminyltransferase-1 knockdown mice displayed reduced CSPG expression, and recovered more completely from SCI than both wild-type mice or chondroitinase treated mice [125]. Thus, strategies targeting CSPG expression are particularly interesting as potential therapeutics for the treatment of SCI.

1.6 Existing therapies for SCI

Currently, there are remarkably few treatments for SCI. Spinal cord decompression is widely practiced as a surgical intervention designed to relieve the physical pressure applied to a compressed spine and to reduce secondary damage. Spinal decompression leads to improved neurological function, reduced hospital stay, fewer secondary complications, earlier mobilization, and quicker transfer to rehabilitation centers [126-130]. However, the decompression surgery needs to occur immediately after SCI as studies have demonstrated that no significant neurologic benefit was seen when spinal cord decompression was performed just over 24 hours post-injury (mean 1.8 days) or during a more chronic time point (mean, 16.8 days) [131].
A pharmacological treatment for SCI also exists; methylprednisolone (MP) has been used as an approved anti-inflammatory clinical treatment for SCI due to its promising immunosuppressive effects [132] and profound anti-oxidant properties [133] in pre-clinical models. However, MP treatment is not without its detractors. In 1979 a multicenter, randomized, double-blinded clinical trial referred to as National Acute Spinal Cord Injury Study (NASCIS) analyzed 330 patients treated with MP or placebo and found no beneficial effect of MP treatment on neurological recovery, motor function, or sensory function, at 6 weeks or 6 months after injury [134]. However, animal studies suggested that the MP dose used in NASCIS I was not sufficient to confer neuroprotection [135, 136]. Thus, a second multicenter trial (NASCIS II) was initiated in 1985 using a higher MP dose, and 487 participants. The administration of a higher dose of MP within 8 hours after injury was associated with a statistically significant improvement in motor and sensory function at the 6-month follow up compared with patients receiving placebo or MP at later time-points [137]. However, there were no functional outcome measures designed to assess whether the statistical improvements noted with MP treatment were indeed clinically relevant. As a whole, this trial was criticized for methodological, scientific, and statistical design issues [138-141]. These criticisms resulted in the development of a third NASCIS trial. The study began in 1991 and followed 499 patients, this time also assessing self-care, mobility, locomotion, sphincter control, communication and social cognition in acute SCI patients. Any benefit of MP treatment was only observed at the 6 week and 6 month follow-ups and no positive effect was noted beyond 1 year [142, 143]. Importantly, all NASCIS trials reported a statistically significant increase in adverse side effects, including: infections, gastrointestinal hemorrhages, sepsis, pulmonary embolism, severe pneumonia, and death [140, 141].
In 2002, the American Association of Neurological Surgeons/Congress of Neurological Surgeons (AANS/CNS) released a statement indicating that the harmful side effects of MP treatment outweighed the potential for clinical benefit [144]. In Canada, administration of MP was common practice following NASCISII (administered in 76% of acute SCI cases) and, following the AANS/CNS recommendations, Canadian clinicians dramatically reduced MP usage for treatment of SCI (administered in only 24% of acute SCI) [145]. As MP is losing favor among clinicians treating SCI, and spinal decompression intervention must occur in the very acute stages after injury, there is clearly a need to develop new efficacious treatments for SCI.

1.7 \textit{Sox9} was identified as a potential regulator of CSPG biosynthesis

Given that CSPGs are such a potent obstacle to regeneration post-SCI the regulation of XT-I, XT-II and C4ST, the enzymes that synthesize the chondroitin sulfate side chains on CSPGs, was investigated. Following SCI, XT-I, XT-II and C4ST all show similar temporal patterns of up-regulated gene expression [146], and are followed by the detection of increased CSPG levels [146]. As the formation of the glial scar is a process that does not occur in the undamaged adult spinal cord, it seemed reasonable to presume that a genetic program for the elaboration of the glial scar would be activated post-SCI to up-regulate the expression of CSPGs and other relevant genes. Thus, researchers in the Brown laboratory conducted an \textit{in silico} phylogenetic footprinting analyses [147] to identify transcription factors that could potentially activate XT-I, XT-II, and C4ST expression. Genomatix software identified five transcription factors which have putative binding sites within the promoters of human, rat, and mouse, XT-I, XT-II and C4ST. Of the five, \textit{Sox9} was particularly interesting as it plays a key role in astrocyte
development [148, 149] and positively regulates the expression of proteoglycans during chondrogenesis in the periphery [150-152].

1.8 Transcription factor Sox9

SOX (SRY-type HMG box) transcription factors are part of a DNA-binding protein subfamily with a high-mobility-group (HMG) domain [153]. Individual members of the SOX family show greater than 50% identity in their HMG domain to SRY, the testes-determining factor, and thus it is not surprising that SOX transcription factors play roles in sex determination [153, 154]. However, SOX transcription factors are involved in a wide range of developmental processes beyond sex determination, including neurogenesis, hematopoiesis, lens development, and skeleton formation [153, 155-161].

Sox9 has two essential functions: it regulates the activity of genes required for testes development, and it regulates essential genes required for cartilage and bone development. Mutations to SOX9 have significant consequences for those afflicted. Disrupting Sox9 results in sex reversal, in which a genetically male (XY) individual will appear phenotypically female as Sox9 is unable to carry out its normal role in testes development [162, 163]. Mutations to Sox9 also result in a genetically dominant skeletal condition known as campomelic dysplasia that presents alongside sex reversal [162, 163]. It is characterized by bone and cartilage abnormalities causing short arms and legs, short digits, bowing of the legs, dislocated hips, feet that are abnormally rotated, and small chest size. The condition is life threatening during the newborn period, with death often resulting from breathing problems as a result of underdeveloped chest or lungs [164]. A few individuals have survived with campomelic dysplasia past infancy. As they
age they develop further orthopedic problems and hearing loss [165, 166]. Mutations to the regions near the SOX9 locus may disrupt enhancer elements that normally regulate the activity of Sox9. If this leads to reduced Sox9 activity an inability to properly control the development of facial structures known as isolated Pierre Robin sequence may result [167]. These skeletal abnormalities result from the individual’s inability to form proper cartilaginous structures due to Sox9 mutation. Sox9’s role in cartilage development is particularly important to this study as proteoglycans are required for proper cartilage formation.

1.9 The role of Sox9 in cartilage formation

Cartilage formation is an essential process during development. Cartilages do not only constitute permanent skeletal structures in the respiratory tract, articular joints, and other organs, but are also essential templates for the formation of endochondral bones. Cartilage is created by chondrocytes secreting specific extracellular matrix components such as various collagens and proteoglycans. Sox9 is expressed in all chondroprogenitors and differentiated active chondrocytes [168]. In vitro studies have shown that Sox9 binds and activates chondrocyte-specific enhancer elements for the Col2a1, Col11a2, Aggrecan, and CD-RAP genes in vitro, demonstrating that these genes are targets of Sox9 [169-172]. Sox9 can also directly activate type 2 collagen expression when ectopically expressed in some non-cartilaginous sites in transgenic mice [173].

Studies using mouse embryo chimeras derived from Sox9 knockdown embryonic stem (ES) cells demonstrated that Sox9 null mutant cells were excluded from chondrogenic mesenchymal condensations and did not express chondrocyte-specific markers for collagen
expression or aggrecan proteoglycan expression [174]. To elucidate further the role of Sox9 in chondrogenesis and chondrocytic differentiation, Akiyama et al. [175] developed a Sox9 conditional knockdown line using the Cre recombinase system. Embryos, in which Sox9 was deleted after mesenchymal condensations, exhibited a severe generalized chondrodysplasia. Most cells were arrested as condensed mesenchymal cells and did not undergo overt differentiation into chondrocytes. Furthermore, chondrocyte proliferation was severely inhibited and joint formation was defective. Embryos missing Sox9 from undifferentiated mesenchymal cells of limb buds displayed the complete absence of both cartilage and bone, and developed chondrodysplasia. This was associated with dramatically reduced expression of Runx2, a transcription factor needed for osteoblast differentiation, as well as an absence of Sox5/Sox6 expression [175]. Sox5 and Sox6 work alongside Sox9 to play a crucial role in chondrocytic differentiation [176]. Sox5 and Sox6 are co-expressed with Sox9 in all chondroprogenitors and all differentiated chondrocytes, and cooperate with Sox9 to activate the Col2a1 enhancer and the Col2a1 gene [177]. Sox9’s involvement in the control of so many genes that contribute to expression of the extracellular matrix, including collagen and proteoglycan genes, suggested that Sox9 may be a master regulator of many genes required for scar formation. Thus, Sox9 was chosen as the first transcription factor to be investigated in search of a putative CSPG modulator in glial scarring post-SCI.
110 Sox9 modulates enzymes essential for CSPG production

Researchers in the Brown laboratory have carried out chromatin immunoprecipitation (ChIP) assays to determine whether Sox9 directly binds XT-I, XT-II and C4ST. Mouse genomic DNA from developing testes that express Sox9 was immunoprecipitated with an anti-SOX9 antibody. The Sox9-immunoprecipitated DNA was purified and subsequently amplified by PCR using primers that flank the Genomatix predicted Sox9 binding sites in the XT-I, XT-II, and C4ST promoters. The ChIP experiments demonstrate in vivo binding of Sox9 to the XT-I and C4ST promoters. Experiments are ongoing to identify Sox9 binding sites in the XT-II promoter region.

To investigate the effects of Sox9 on astrocyte-produced XT-I, XT-II, and C4ST, a primary rat astrocyte tissue culture model was used. Over-expression of Sox9 was accomplished by transient transfection of a Sox9 expression cassette and demonstrated increased astrocyte XT-I, XT-II, and C4ST expression. Sox9 knockdown in primary rat astrocyte cultures, accomplished by treatment with anti-Sox9 siRNA, demonstrated decreased astrocyte XT-I, XT-II, and C4ST expression along with increased laminin and fibronectin expression [146]. These findings suggested that Sox9 plays a role in up-regulating anti-regenerative CSPG production as well as down-regulating pro-regenerative laminin and fibronectin expression [146].

A second, seemingly unrelated, experimental strategy to promote recovery post-SCI, is the intravenous administration of anti-CD11d monoclonal antibody. This anti-inflammatory treatment that blocks leukocytes from infiltrating into the damaged spinal cord has been shown to decrease lesion size, improve neurological recovery after rodent SCI, and indirectly result in reduced levels of Sox9 at the lesion epicenter [178]. The observed reduction in Sox9 was
accompanied by a reduction in XT-I, XT-II, and C4ST expression as well as an increase in laminin and fibronectin expression [179]. Consistent with these mRNA changes, CSPGs were reduced by approximately one half in the lesions of anti-CD11d-treated rats, and laminin was increased. These results are in keeping with our previous observation that pro-inflammatory cytokines (IL-6, TGF-β2 and PDGF) up-regulate the expression of SOX9 [146]. We conjectured that these changes in Sox9, CSPG, and laminin/fibronectin expression in the scars of anti-CD11d mAb-treated rats greatly contributed to the increase in axonal growth and sprouting as well as functional recovery observed [178]. We were thus interested in investigating CSPG expression, recovery of hind limb motor function, and neuroplasticity in Sox9 knockdown mice.

1.11 The dorsal contusion spinal cord injury model used in these studies

The SCI model used in this dissertation is a thoracic spinal cord dorsal contusion. Mice are anesthetized, restrained in a mechanical device which stabilizes their spinal cord, and a laminectomy is performed to expose the 9th thoracic spinal segment. A computer controlled injury is induced by the Infinite Horizons Impactor mechanically delivering a blunt contusion to the exposed spinal cord (without disruption of the dura) [180]. The device records precise force delivered and displacement of spinal tissue, thus allowing for consistent and reproducible injuries. This contusion model of SCI produces primary mechanical trauma to the cord as well as significant secondary damage [181]. The resulting injury generates motor and behavioral deficits similar in morphology and pathology to common human SCI [182, 183]. Following SCI, motor function is evaluated using the Basso Mouse Scale (BMS) for scoring hind limb function [184] as well as locomotor activity box for assessment of total locomotion over a given period of time.
1.12 Conditional Sox9 knockdown mouse breeding strategy

Conventional Sox9 knockdown embryos have been generated but are unsuitable for studies of SCI as Sox9 knockdown embryos do not survive to birth [174, 185]. Thus, to study the effect of Sox9 knockdown in adult mice which develop with normal Sox9 activity, we bred two existing mouse strains together. The first mouse strain used was Sox9<sup>flox/flox</sup>, a transgenic strain carrying floxed Sox9 alleles (exons 2 and 3 of Sox9 surrounded by loxP sites) which has been used successfully for the conditional knockdown of Sox9 in various cell types [148, 175]. The second mouse strain used was CAGGCre-ER, a transgenic line that ubiquitously expresses Cre recombinase fused to the mutated ligand binding domain of the mouse estrogen receptor under the control of the chicken beta actin promoter/enhancer coupled to the Cytomegalovirus (CMV) immediate early enhancer [186]. The mutated estrogen receptor ligand binding domain does not bind endogenous estradiol but rather binds to exogenously administered tamoxifen. Cre recombinase expressed in these animals is trapped outside the nucleus by the mutated estrogen receptor until tamoxifen administration releases Cre allowing for its transport to the nucleus where it excises floxed regions of DNA. By breeding the Sox9<sup>flox/flox</sup> mice to the CAGGCre-ER mice we generate Sox9<sup>flox/flox</sup>;CAGGCre-ER offspring (Sox9<sup>flox/flox</sup>;Cre). The F1 pups of Sox9<sup>flox/flox</sup>;Cre genotype were backcrossed with the Sox9<sup>flox/flox</sup> mice for more than 5 generations to attain the Sox9<sup>flox/flox</sup>;Cre and their Sox9<sup>flox/flox</sup> littermates described herein. In these Sox9<sup>flox/flox</sup>;Cre mice, tamoxifen administration allows us to examine the molecular, cellular, and neurological responses to SCI in the presence of greatly reduced expression levels of Sox9. In all of our studies Sox9<sup>flox/flox</sup> littermates that do not carry the CAGGCre-ER allele serve as controls as they express normal levels of Sox9 (even after tamoxifen administration). These Sox9
conditional knockdown (Sox9\textsuperscript{floxflox};Cre) and control mice expressing normal levels of Sox9 (Sox9\textsuperscript{floxflox}) were used to evaluate the following specific goals for this thesis.

1.13 Specific goals

This thesis had three major goals: 1) to determine if the transcription factor Sox9 controls chondroitin sulfate proteoglycan gene expression, 2) to determine if Sox9 conditional knockdown mice display improved hind limb functional recovery post-SCI, and 3) to determine if Sox9 conditional knockdown mice display increased neuroplasticity post-SCI.

I set out the following seven objectives to investigate these goals:

1) To breed Sox9 conditional knockdown mice for use in studying the effect of Sox9 knockdown on recovery post-SCI in the mouse.

2) To determine if Sox9 conditional knockdown mice display decreased CSPG expression in \textit{vitro}.

3) To determine if Sox9 conditional knockdown mice display decreased CSPG expression in \textit{vivo}.

4) To determine if Sox9 conditional knockdown mice display increased hind limb function post-SCI.

5) To determine if Sox9 conditional knockdown mice display increased locomotion post-SCI.
6) To determine if Sox9 conditional knockdown mice display increased axonal regeneration post-SCI.

7) To determine if Sox9 conditional knockdown mice display increased synapse plasticity post-SCI.

The first objective was accomplished by breeding Sox9\textsuperscript{flax/\textit{flox}} mice to CAGGCre-ER mice to generate Sox9\textsuperscript{flax/\textit{flox};\textit{Cre}} and their Sox9\textsuperscript{flax/\textit{flox}} littermates. On tamoxifen administration the Sox9\textsuperscript{flax/\textit{flox};\textit{Cre}} mice lose expression of Sox9 while the Sox9\textsuperscript{flax/\textit{flox}} littermates will still express wild type levels of Sox9\textsuperscript{flax/\textit{flox}}. By comparing Sox9\textsuperscript{flax/\textit{flox};\textit{Cre}} and Sox9\textsuperscript{flax/\textit{flox}} littermates we can ascertain the effect of Sox9 knockdown on mouse physiology post-SCI. The second objective was accomplished by using real time PCR to monitor mRNA changes in Sox9 knockdown and control primary astrocyte cultures. The third objective was accomplished by using western blot and immunohistochemistry to monitor protein changes in Sox9 knockdown and control mice. The fourth objective evaluated hind limb functional recovery in both a proof of principle model, as well as a more clinically relevant delayed knockdown model. Hind limb functional recovery from a T9 dorsal contusion SCI was evaluated in both models by way of Basso mouse scale hind limb function scoring. The fifth objective was accomplished by use of rodent activity box locomotion testing performed on Sox9 knockdown and control mice in both a proof of principle model, as well as a more clinically relevant delayed knockdown model. The sixth objective was accomplished by neuronal labeling techniques including retrograde labeling to assess for sparing and long range regeneration of axons through the injury site, and anterograde labeling to assess for axonal sprouting caudal to the injury site. The seventh and final objective was investigated using synapse markers to assess synaptic plasticity.
1.14 Summary

The failure of CNS axons to undergo significant regeneration following injury is considered to be the main reason why most animals do not display significant functional recovery post-neurotrauma. One of the most detrimental causes of this failure of CNS axons to undergo significant regeneration is the expression of CSPG extracellular matrix post-injury. We have previously identified Sox9 as a transcription factor which may up-regulate expression of this anti-regenerative CSPG extracellular matrix. This thesis will attempt to determine if Sox9 ablation will inhibit CSPG extracellular matrix production both \textit{in vitro} and \textit{in vivo}, result in increased neuroplasticity and axonal regeneration, and lead to improved hind limb motor function post-SCI. \textbf{Chapter 2} of this thesis contains our proof of principle study in which we show that, following SCI, Sox9 knockdown mice display reduced CSPG expression and improved hind limb functional recovery. \textbf{Chapter 3} of this thesis details a more clinically relevant injury model in which Sox9 is knocked down approximately 2 weeks post-injury, and still results in reduced CSPG expression and improved hind limb functional recovery. \textbf{Chapter 4} of this thesis investigates the neuronal mechanism behind this improved hind limb functional recovery and finds evidence for increased neuroplasticity, revealed as reactive sprouting and increased synaptic plasticity, in Sox9 knockdown mice post-SCI.
1.15 References


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Chapter 2: Conditional SOX9 ablation reduces chondroitin sulfate proteoglycan expression and improves motor function following spinal cord injury

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Running title: SOX9 knockdown improves recovery after SCI

Keywords: SOX9, spinal cord injury, neuroplasticity, CSPG, regeneration, perineuronal nets
2.0 Abstract

Chondroitin sulfate proteoglycans (CSPGs) found in perineuronal nets and in the glial scar after spinal cord injury have been shown to inhibit axonal growth and plasticity. Since we have previously identified SOX9 as a transcription factor that up-regulates the expression of a battery of genes associated with glial scar formation in primary astrocyte cultures, we predicted that conditional *Sox9* ablation would result in reduced CSPG expression after spinal cord injury and that this would lead to increased neuroplasticity and improved locomotor recovery. Control and *Sox9* conditional knockdown mice were subject to a 70 kdyne contusion spinal cord injury at thoracic level 9. One week after injury, *Sox9* conditional knockdown mice expressed reduced levels of CSPG biosynthetic enzymes (XT-1 and C4st), CSPG core proteins (brevican, neurocan and aggrecan), collagens 2a1 and 4a1, and GFAP, a marker of astrocyte activation, in the injured spinal cord compared to controls. These changes in gene expression were accompanied by improved hind limb function and locomotor recovery as evaluated by the Basso Mouse Scale (BMS) and rodent activity boxes. Histological assessments confirmed reduced CSPG deposition and collagenous scarring at the lesion of *Sox9* conditional knockdown mice, and demonstrated increased neurofilament-positive fibers in the lesion penumbra and increased serotonin immunoreactivity caudal to the site of injury. These results suggest that SOX9 inhibition is a potential strategy for the treatment of SCI.
2.1 Introduction

Damaged axons have a limited capacity for regeneration following adult mammalian spinal cord injury (SCI) [1]. This limited capacity for repair has been attributed, in part, to the nonpermissive environment of the glial scar that forms in the penumbra surrounding the lesion site [2-6]. This glial scar is predominantly formed from extracellular matrix (ECM) molecules expressed by reactive astrocytes although macrophages, microglia, oligodendrocytes, invading Schwann cells and meningeal fibroblasts all contribute to production of the scar matrix [6]. Chief of the many ECM molecules that serve to inhibit axonal regeneration are the chondroitin sulfate proteoglycans (CSPGs) [7, 8] that have greatly increased expression following SCI [4, 9]. CSPGs are a class of ECM macromolecules that share a common structure composed of a central core protein and a number of chondroitin sulfate side chains [10]. Both in vitro and in vivo studies have shown that axons do not extend into CSPG-rich ECM [4, 5, 11-13], and specific CSPGs which inhibit neurite outgrowth have been identified including: aggrecan [14], neurocan [15], phosphocan [16], brevican [17], versican [18], and NG2 [19].

Strategies designed to target CSPGs at the spinal lesion have resulted in improved axonal regeneration after SCI. Enzymatic digestion of the chondroitin sulfate side chains, found on all CSPGs, by intrathecal chondroitinase treatment resulted in increased regeneration of ascending and descending tracts after SCI [20]. The combination of chondroitinase ABC with peripheral nerve grafts [21, 22], rehabilitation [23, 24], or neural precursor cell transplantation [25] have all led to improved axonal regeneration and recovery.

We have previously argued that genes with related function are regulated together as classes or batteries after SCI [26] and that, in astrocytes, genes that promote axon regeneration
and genes that inhibit axon regeneration would be regulated as gene classes. We predicted that the transcription of genes involved in CSPG production, the genes encoding CSPG core proteins, and genes encoding the enzymes responsible for generating the chondroitin sulfate side chains such as xylosyltransferase-I (XT-I), XT-II and chondroitin-4-sulfotransferase-1 (C4st-1), would be coordinately regulated after SCI. Using bioinformatics we identified putative binding sites for the transcription factor SOX9 (sex-determining region Y-box 9) in the promoter sequences of XT-I, XT-II and C4st-1 in rats, mice and humans. We subsequently used gain of function and loss of function experiments to demonstrate that SOX9 positively regulates the expression of XT-I, XT-II, and C4st-1 in primary astrocyte cultures [27]. Thus we hypothesized that conditional ablation of Sox9 in mice would result in reduced expression of CSPGs and improved recovery after SCI. We herein report improved hindlimb locomotor recovery after SCI in a line of conditional Sox9 knockdown mice that correlates with reduced expression of CSPGs and related ECM proteins in the lesion penumbra and at sites more distant to the lesion epicenter.

2.2 Materials and Methods

Mouse breeding and Sox9 conditional knockdown

Conventional Sox9 knockdown mice have been generated but are unsuitable for studies of SCI as both Sox9 knockdown (Sox9<sup>-/-</sup>) and heterozygote (Sox9<sup>+/-</sup>) embryos do not survive to birth [28]. To evaluate SOX9 loss-of-function after SCI, in a nervous system that developed with normal levels of SOX9 activity, a tamoxifen-inducible conditional Sox9 knockdown strategy was used. We bred a mouse strain that carries floxed Sox9 (exons 2 and 3 of Sox9 surrounded by loxP sites) alleles [29] (Sox9<sup>flox/flox</sup>) with a transgenic mouse line that expresses Cre recombinase fused
to the mutated ligand binding domain of the human estrogen receptor (ER) under the control of a chimeric cytomegalovirus immediate-early enhancer/chicken β–actin promoter (B6.Cg-Tg(CAG-Cre/Esr1)5Amc/J)[30] (Jackson Laboratories, Bar Harbor, Maine). The mutated ER ligand binding domain of the fusion protein does not bind endogenous estradiol but is highly sensitive to nanomolar concentrations of tamoxifen [31]. The Cre-ER fusion protein remains trapped in the cytoplasm of all cells until tamoxifen administration allows its transport to the nucleus where it excises loxP-flanked Sox9 DNA [30]. The resulting Sox9\textsuperscript{flox/flox};CAGGCreER (Sox9\textsuperscript{flox/flox};Cre) offspring served as tamoxifen inducible Sox9 knockdown animals, and Sox9\textsuperscript{flox/flox} offspring served as control animals expressing normal levels of SOX9. Animals were genotyped by PCR analysis using the following primers:

Sox9\textsuperscript{flox} allele: 5’-ACACAGCATAGGCTACCTG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

Sox9\textsuperscript{wildtype} allele: 5’-GGGGCTTGTCTCCTTCAGAG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

Sox9\textsuperscript{knockdown} allele: 5’-GTCAAGCGACCCATG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

Cre\textsuperscript{+} allele: 5’-CAATTGACTGACGTAC-3’ and 5’-AGCTGGGCCAAATGTTGCTG-3’.

Tamoxifen (Sigma Aldrich, St. Louis, Missouri) was administered at 3 mg/20 g mouse by oral gavage to all Sox9\textsuperscript{flox/flox};Cre and Sox9\textsuperscript{flox/flox} littermates once per day for 7 days. Following the final day of tamoxifen oral gavage, the animals were housed for 7 days without treatment to allow time for Cre-mediated recombination and tamoxifen clearance prior to subsequent SCI.
Primary astrocyte culture

Primary astrocyte cultures were prepared from newborn $\text{Sox9}^{\text{flox/flox};\text{Cre}}$ or $\text{Sox9}^{\text{flox/flox}}$ control mice at postnatal day 1. The upper portion of the skull was removed and the meninges carefully dissected away to avoid contamination of the culture with fibroblasts. The neocortices were removed, individually placed into serum-free Eagle Minimum Essential Medium (EMEM) (Lonza, Walkersville, Maryland), homogenized by trituration, and gravity-filtered through a 40-μm cell strainer (Becton Dickinson and Company, Toronto, Ontario). The cells were plated in EMEM + 20% FBS (Invitrogen, Carlsbad, California), penicillin/streptomycin (Invitrogen, Carlsbad, California); each animal’s cells were divided into two wells each of a 6-well dish (Becton Dickinson and Company, Toronto, Ontario). After 2 days, media was changed to EMEM + 10% FBS, penicillin/streptomycin, and was changed three times per week thereafter. After 2 weeks in culture 1 μM 4-hydroxytamoxifen (Sigma Aldrich, St. Louis, Missouri) was administered in three changes of media over 1 week. Following 4-hydroxytamoxifen administration the cells were cultured in normal media for 1 more week. The percentage of GFAP-expressing cells in these cultures was found to be >95%.

Real time PCR

RNA was extracted from $\text{Sox9}$ conditional knockdown and $\text{Sox9}$ positive control primary astrocyte cultures 1 week post tamoxifen administration, and from the lesion epicentre of $\text{Sox9}$ conditional knockdown and $\text{Sox9}$ positive control mice 1 week post-SCI, using the RNA-Easy kit according to the manufacturer's instructions (Qiagen, Valencia, California). First strand cDNA was synthesized from 1 μg RNA per sample using the High Capacity cDNA Archive Kit.
according to the manufacturer instructions (Applied Biosystems, Carlsbad, California). The
primer probe sets, optical adhesive covers, and PCR plates were purchased from Applied
Biosystems (Carlsbad, California). All primer probes were labeled with 5'FAM and with
3'TAMRA as quencher with the exception of the 18s ribosomal probe, which was labeled with 5'
VIC. TaqMan assays were conducted using the Applied Biosystems gene expression assay
primer probe sets listed in Table 1.

Table 1. List of TaqMan Real-Time PCR Primer Probe Sets

<table>
<thead>
<tr>
<th>Primer Probe</th>
<th>Catalog number</th>
<th>PCR Ct range</th>
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<tbody>
<tr>
<td>18s</td>
<td>4308329</td>
<td>14.72–16.07</td>
</tr>
<tr>
<td>Sox9</td>
<td>Mm00448840_m1</td>
<td>21.88–23.64</td>
</tr>
<tr>
<td>XT-I</td>
<td>Mm00558690_m1</td>
<td>27.34–29.57</td>
</tr>
<tr>
<td>XT-II</td>
<td>Mm00461181_m1</td>
<td>24.72–25.87</td>
</tr>
<tr>
<td>C4st-1</td>
<td>Mm00517563_m1</td>
<td>21.72–23.01</td>
</tr>
<tr>
<td>Aggreecan</td>
<td>Mm00545807_m1</td>
<td>24.53–27.07</td>
</tr>
<tr>
<td>Brevican</td>
<td>Mm00476090_m1</td>
<td>18.35–21.70</td>
</tr>
<tr>
<td>Neurocan</td>
<td>Mm00484007_m1</td>
<td>17.31–20.73</td>
</tr>
<tr>
<td>Collagen 2A1</td>
<td>Mm01309562_g1</td>
<td>23.40–27.33</td>
</tr>
<tr>
<td>Collagen 4A1</td>
<td>Mm00802377_m1</td>
<td>20.73–21.39</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mm01253033_m1</td>
<td>19.96–22.16</td>
</tr>
<tr>
<td>Cartilage link protein</td>
<td>Mm00488952_m1</td>
<td>28.35–29.94</td>
</tr>
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</table>
TaqMan (Applied Biosystems, Carlsbad, California) gene expression assays were conducted on a 7900HT fast real time PCR apparatus (Applied Biosystems, Carlsbad, California) using thermal cycler conditions set as follows; 10 min at 95°C followed by 40 cycles of 30 s at 95°C followed by 30 s at 60°C. Cycle thresholds (Ct) for all target genes were kept below 30 as indicated in Table 1. A standard curve of cycle thresholds using cDNA serial dilutions was established and used to calculate mRNA expression. Target gene mRNA expression was normalized to the amount of 18S mRNA present in each sample. The ratio of knockdown to control sample normalized target gene mRNA was analyzed by Student's T-test.

**Western blotting**

Protein was isolated from the lesion site (0.45 cm) in tamoxifen-treated Sox9 conditional knockdown and Sox9 positive control mice 2 weeks post-SCI. The spinal cord tissue was lysed in modified RIPA buffer (1% nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA, pH 7.5, plus 1 complete Mini protease inhibitor tablet/7mL RIPA buffer (Roche Molecular Biochemicals, Indianapolis, Indiana) on ice using a ground glass homogenizer. The protein mixture was centrifuged at 13,000 x g for 5 min and the supernatant collected, and diluted in reducing PAGE loading buffer. Protein samples were loaded on reducing SDS-PAGE gels at 10 μg/well. The membrane was blocked in 10% nonfat powdered milk and then incubated with primary antibodies; anti-SOX9 (AB 5535, Millipore, Billerica, Massachusetts used at 1:1000), anti-GFAP (MAB360, Millipore, Billerica, Massachusetts, used at 1:1000), and anti-β-actin (A1978, Sigma, St. Louis, Missouri, used at 1:10,000) for protein
expression assessed by western blot. HRP conjugated anti-mouse IgG (715-035-151, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and HRP conjugated anti-rabbit IgG (711-035-152, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) secondary antibodies were used at 1:20,000 dilution to detect SOX9, GFAP, and β-actin protein expression. SOX9 and GFAP protein expression was normalized to β-actin protein expression by densitometry using the EpiChemi³ Darkroom (UVP Bioimaging Systems, Upland, California) and LabWorks software (Media Cybernetics Inc, Bethesda, Maryland). Protein samples were also loaded in parallel at 3 μg/well into a Bio-Rad Slot-blot apparatus (BioRad, Mississauga, Ontario) and vacuum transferred onto a nitrocellulose membrane (BioRad, Mississauga, Ontario). The membrane was blocked in 10% nonfat powdered milk and then incubated with primary antibody at 1:200 dilution overnight for anti-CS-56 (C8035, Sigma, St. Louis, Missouri) or 1:10,000 dilution for anti-β-actin (A1978, Sigma, St. Louis, Missouri). HRP conjugated anti-mouse IgM (62-6820, Invitrogen, Carlsbad, California) and anti-mouse IgG (715-035-151, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) secondary antibodies were used at 1:20,000 dilutions to detect CS56 and β-actin protein expression. CS56 protein expression was normalized to β-actin protein expression by densitometry using the EpiChemi³ Darkroom (UVP Bioimaging Systems, Upland, California) and LabWorks software (Media Cybernetics Inc, Bethesda, Maryland).
Spinal cord injury

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the Guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. One week after the last tamoxifen oral gavage, 13 female $Sox9^{flx/flx;Cre}$ and 16 female $Sox9^{flx/flx}$ mice were anesthetized with 100 mg/kg ketamine: 5 mg/kg xylazine. The T9 spinal cord segment was exposed by a dorsal laminectomy. The spinal cord was stabilized at T7 and T9 with forceps. The T9 spinal segment was injured by a 70 kdyne contusion delivered with a 1s dwell time by computer controlled Infinite Horizons Impactor (displacement range: 500-900 $\mu$M) (Precision Systems and Instrumentation, Fairfax, Virginia). Following SCI the mice were housed individually. Baytril (25 mg/kg, Bayer, Toronto, Ontario, Canada) and buprenorphine (0.01 mg/kg, Schering-Plough, Hertfordshire, UK) were injected subcutaneously for 3 days post-SCI. Bladders were manually emptied twice daily for the duration of the experiment.

Behavioral testing

Locomotor recovery of the animals was assessed by two blinded observers using the Basso Mouse Scale (BMS) open field locomotor score [32]. The day following SCI, all mice were evaluated for any signs of locomotor recovery in their hindlimbs and mice that had BMS scores $> 0.5$ were excluded from further analyses (4 $Sox9$ conditional knockdowns and 5 controls). Animals were evaluated once per week for 14 weeks after SCI. Left and right hind limb scores were averaged to generate a composite score. In addition, locomotion was evaluated at 14 weeks post-SCI by rodent activity box (Accuscan Instruments Inc, Columbus, Ohio). The
activity box records distance traveled by detecting breaks in a series of infrared light beams. The total distance the mice traveled was measured over a 2 hour period at night (during their normal awake circadian cycle).

**Spinal cord sectioning**

Protein expression levels of SOX9 target genes were assessed at 14 weeks post-SCI. Animals were deeply anesthetized with 100 mg/kg ketamine: 5 mg/kg xylazine, and cardiac perfusion was carried out with 20 ml of saline at pH 7.4 followed by 20 ml 4% paraformaldehyde (4% PFA in 0.1 M phosphate buffer at pH 7.4). Spinal cords were dissected and post-fixed for 2 h in 4% PFA followed by cryoprotection in 20% sucrose in 0.1 M phosphate buffer at pH 7.4 at 4°C overnight. Spinal cords were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A. Inc, Torrance, California), frozen over dry ice, and stored at -80°C overnight. Frozen cords were then cross-sectioned at 16 µm using a cryostat, and serially thaw-mounted on Superfrost™ glass slides (Fisher Scientific Company, Ottawa, Canada).

**Immunohistochemistry and trichrome staining**

Immunohistochemistry was conducted using the primary antibodies listed in Table 2. Cryosectioned slides were rinsed in PBS and treated with 5% normal goat serum and 0.1% triton-X-100 in PBS at room temperature for 1 h.Slides were incubated with the appropriate dilutions of primary antibodies in a humidified chamber at 4°C overnight. Sections were stained for CSPG expression using the monoclonal antibody CS56 that recognizes the terminal portions
of chondroitin sulfate-4 or -6 side chains and thus detects a variety of CSPGs [33] and a 
biotinylated goat anti-mouse IgM (Vector laboratories, Burlingame, California) secondary 
antibody (1:200). Sections were then incubated for 45 min with avidin-peroxidase conjugate 
(Elite Kit, Vector laboratories, Burlingame, California) at room temperature, and the signal 
visualized by peroxidase diaminobenzine (DAB, Invitrogen, Carlsbad, California). Sections to be 
stained for perineuronal nets (PNNs) were washed in PBS 3 x 10 min, and incubated with 
biotinylated Wisteria Floribunda Lectin (WFA, Sigma Aldrich, St. Louis, Missouri) (1:1000) for 
1 h at room temperature. Sections were then incubated for 45 min with avidin-peroxidase 
conjugate (Elite Kit, Vector laboratories, Burlingame, California) at room temperature, and the 
signal visualized by peroxidase diaminobenzine (DAB, Invitrogen, Carlsbad, California). All 
DAB staining was conducted with a 2 min DAB reagent incubation time for all Sox9 conditional 
knockdown and control cord sections, and were completed at the same time. Immunofluorescent 
labeling of the remaining proteins was performed using the following secondary antibodies; 
Alexa-Fluor 488-conjugated goat anti-mouse IgG (1:500, Invitrogen, Carlsbad, California), 
Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:500, Invitrogen, Carlsbad, California), or 
Alexa-Fluor 594-conjugated goat anti-rabbit IgG (1:500, Invitrogen, Carlsbad, California), for 1 
h at room temperature. Slides were then washed in PBS and coverslips were attached with 
ProLong Gold Anti-Fade mounting medium (Invitrogen, Carlsbad, California). Gomori’s 
Trichrome staining was used to stain for collagen according to the manufacturer’s instructions 
(HT10316, Sigma Aldrich, St. Louis, Missouri).
Table 2. List of primary antibodies and stains used for spinal cord staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFAP</td>
<td>1:500</td>
<td>Mouse IgG</td>
<td>Millipore, Billerica, Massachusetts</td>
</tr>
<tr>
<td>Anti-CS56</td>
<td>1:300</td>
<td>Mouse IgM</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
</tr>
<tr>
<td>Anti-NF200</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
</tr>
<tr>
<td>Anti-5HT</td>
<td>1:500</td>
<td>Rabbit IgG</td>
<td>ImmunoStar, Hudson, Wisconsin</td>
</tr>
<tr>
<td>WFA</td>
<td>1:1000</td>
<td></td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
</tr>
</tbody>
</table>

Quantification of GFAP, CS56, trichrome, NF-200, 5-HT, and WFA staining

GFAP, CS56, trichrome, and NF-200 staining were analyzed as follows. Cross-sections 16 μm thick and 160 μm apart between 1.6 mm rostral through 1.6 mm caudal to the epicentre of injury were analyzed for positive staining using Image Pro Plus software (Media Cybernetics Inc, Bethesda, Maryland). A threshold was set for each stain that identified positive signal (staining above background levels). The area of positive staining was normalized to total cord area. Staining results were grouped into 5 bins based on position relative to the lesion epicentre that was arbitrarily set as zero. The bin set as the epicenter encompassed 0.65 mm rostral and 0.65 mm caudal to the epicenter. The 1.3 mm included in the “epicenter” bin that approximates the size of the head of the impactor. The rostral bins encompassed two directly adjacent segments of spinal cord rostral to the lesion (0.8 mm each) and the two caudal bins encompassed two directly adjacent segments of spinal cord rostral to the lesion (0.8 mm each).
The area of 5-HT immunoreactivity (area per area of interest) was quantified in the intermediolateral cell column (IML) and in the ventral horns in 16 μm thick cross-sections 160 μm apart obtained 0.8-1.6 mm caudal to the injury site using Image Pro Plus Software (Media Cybernetics Inc, Bethesda, Maryland). A single pre-set area was used to define all IML or ventral horn regions in all cords across both Sox9 conditional knockdown and control animal sections. The area of positive 5-HT immunoreactivity was quantified within this set area defined as the IML or ventral horn. The area of WFA immunoreactivity to identify PNNs was analyzed using 16 μm thick cross-sections 160 μm apart sampled at T10. Positive staining was quantified using Image Pro Plus Software (Media Cybernetics Inc, Bethesda, Maryland) using a threshold which identified positive signal (staining above background levels).

**Statistical analysis**

Mean values are expressed ± SE. Both *in vitro* and *in vivo* mRNA analyses were subjected to statistical analysis using Student’s T-test. 5-HT and WFA quantification was subjected to statistical analysis using Student’s T-test. CS56, trichrome, GFAP, and NF-200 immunohistochemical quantification was subjected to statistical analysis using two-way ANOVA with Neuman-Keuls post-hoc test at each binned region of the cord, rostral, epicenter, and caudal to the site of injury. BMS results were subjected to statistical analysis using two-way repeated measures ANOVA with Neuman-Keuls post-hoc test. Activity Box locomotion was subjected to statistical analysis using one-way ANOVA with Neuman-Keuls post hoc test. Analyses were conducted with GraphPad Prism software (GraphPad Software Inc, La Jolla, California), except for two-way ANOVAs which were conducted with SigmaStat software.
(Systat Software Inc, San Jose, California), and significance was accepted at p<0.05. A two-way ANOVA summary table is provided as Supplementary Table 1.

2.3 Results

Changes in gene expression in primary astrocytes isolated from Sox9 conditional knockdowns

Astrocyte cultures were isolated from Sox9 conditional knockdown and from control mice to evaluate the effects of Sox9-ablation on gene expression in primary astrocytes. All cultures were treated with 4-hydroxytamoxifen for 1 week and then cultured free of tamoxifen for an additional week before harvesting for RNA isolation. Using quantitative-PCR (Q-PCR) we measured the mRNA levels of Sox9, XT-I, XT-II, C4st-1, Col2a1, Col4a1, cartilage link protein (Crtl), and aggrecan (Agc) (Fig. 1). The mRNA levels of glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, and brevican and neurocan (two CSPG core proteins) were also measured as we predicted that SOX9 would regulate genes broadly associated with astrocyte activation and scar production. Quantitative PCR demonstrated that administration of 4-hydroxytamoxifen resulted in a 72% ± 4% reduction in Sox9 mRNA expression compared to control mouse astrocyte cultures. Reduced Sox9 expression was associated with a statistically significant reduction in the expression of XT-I, Agc, brevican, neurocan, Col2a1, and GFAP, in comparison to control astrocyte cultures (Fig. 1).
Figure 1. Astrocytes from Sox9 conditional knockdown mice demonstrate reduced glial scar gene expression compared to control mice. Treating Sox9^{flox/flox};Cre astrocyte cultures with 1 μM 4-hydroxytamoxifen for one week results in a 72% ± 4% reduction in Sox9 mRNA levels and is accompanied by a statistically significant reduction in XT-1, aggrecan (Agc), brevican (B-can), neurocan (N-can), Col2A1, and GFAP mRNA levels compared to Sox9^{flox/flox} astrocytes treated with 1 μM 4-hydroxytamoxifen, (p<0.05, Student’s T-test; n=4 per group).
Figure 1. Astrocytes from Sox9 conditional knockdown mice demonstrate reduced glial scar gene expression compared to control mice.
Changes in gene expression in the injured spinal cord in Sox9 conditional knockdowns

To determine if the reductions in gene expression observed in the Sox9\textsuperscript{flox/flox};Cre primary astrocyte cultures would also be observed after SCI we evaluated mRNA expression levels at the lesion in Sox9 conditional knockdown and control mouse spinal cords one week after a 70 kdyne SCI. Q-PCR demonstrated a 62% ± 11% reduction in Sox9 mRNA levels in the Sox9 conditional knockdown mice compared to controls (Fig. 2). This reduction in Sox9 mRNA levels was associated with a statistically significant reduction in XT-I, C4st-1, Agc, brevican, neurocan, Col2a1, Col4a1, and GFAP mRNA expression as compared to control mice (Fig. 2). To determine if these changes in mRNA levels result in parallel changes in protein levels we evaluated protein expression by western and slot blot analysis. Sox9 conditional knockdown mice displayed significantly reduced SOX9, GFAP and CSPG protein expression 2 weeks post-SCI (Fig. 3).

Sox9 conditional knockdown mice demonstrate improved locomotor recovery after SCI

As the glial scar in general and CSPGs in particular have been identified as inhibitors of axonal regeneration after SCI we predicted that the reduction in CSPG and collagen expression at the spinal lesion observed at 1-2 weeks post-injury would lead to improved locomotor recovery in Sox9 conditional knockdown mice. Sox9\textsuperscript{flox/flox};Cre and control mice were administered tamoxifen for one week and allowed a week for tamoxifen washout before undergoing a 70 kdyne SCI using the Infinite Horizon impactor. Hind limb function was evaluated weekly for 14 weeks post-SCI. On day one following SCI all mice displayed paralyzed hind limbs, scoring a zero on the Basso Mouse Scale (BMS). In both Sox9
Figure 2. *Sox9* conditional knockdown mice demonstrate reduced glial scar gene expression compared to control mice. Spinal cord-injured, tamoxifen-treated *Sox9* flox/flox; Cre mice demonstrate a 62% ± 11% reduction in *Sox9* mRNA expression compared to spinal cord-injured, tamoxifen-treated *Sox9* flox/flox mice one week post-SCI. This reduction in *Sox9* expression is associated with a statistically significant reduction in XT-1, C4st-1, Agc, brevican (B-can), neurocan (N-can), Col2A1 and 4A1, and GFAP mRNA levels (p<0.05, Student’s T-test; n=5 per group).
Figure 2. Sox9 conditional knockdown mice demonstrate reduced glial scar gene expression compared to control mice.
Figure 3. Sox9 conditional knockdown mice demonstrate reduced SOX9, GFAP, and CSPG protein 2 weeks post-SCI. A) Western blot analysis and subsequent densitometry (B, C) demonstrate reduced SOX9 and GFAP levels in Sox9 conditional knockdown mice in comparison to control mice (normalized to β-actin levels) (p=<0.05, Student’s T-test; n=3). D) Slot blot and subsequent densitometry (E) demonstrates reduced CSPG expression in Sox9 conditional knockdown mice compared to controls (normalized to β-actin levels) (p=<0.05, Student’s T-test; n=3).
Figure 3. Sox9 conditional knockdown mice demonstrate reduced SOX9, GFAP, and CSPG protein 2 weeks post-SCI.
conditional knockdown and control mice hind limb locomotion gradually improved over time. Locomotor BMS scoring in control mice reached a plateau of 0.63 ± 0.21 at 4 weeks post-SCI, (Fig. 4a). The median BMS score in this group of 0.5 indicates slight (less than 90˚) movement in one ankle. In contrast, the BMS scores of the Sox9 conditional knockdown mice continued to improve past 4 weeks and did not reach a plateau until 11 weeks post-SCI, achieving an average BMS score of 1.81 ± 0.19. The median BMS score for Sox9 conditional knockdown mice of 2 indicates extensive (greater than 90˚) movement in both ankles. The significantly higher scores of the Sox9 conditional knockdown mice were accompanied by a statistically significant increase in ability to achieve plantar placement of their hind limbs (Chi squared test p=0.005); six of nine Sox9 conditional knockdown mice displayed at least one limb capable of plantar placement compared to one of eleven control mice which were capable of plantar placement. Finally, Sox9 conditional knockdown mice and controls were placed in a computer-monitored rodent activity box to record total distance traversed over a 2 h period. Fourteen weeks after SCI, control mice covered a total distance of 1414 ± 269 cm in 2 h, whereas Sox9 conditional knockdown mice covered a total distance of 3481 ± 814 cm in 2 h. The distance traversed by the Sox9 conditional knockdown mice was significantly greater than that in the injured control animals and was not significantly different from uninjured Sox9floxflox mice (3330 ± 402 cm in 2 h) or uninjured control mice (3452 ± 526.5 cm in 2 h) (p=0.027 by 1-way ANOVA with Neuman-Keuls post hoc test) (Fig. 4b).
**Figure 4.** *Sox9* conditional knockdown mice demonstrate improved locomotor recovery after SCI. A) *Sox9* conditional knockdown mice display increased hind limb functional recovery in comparison to control mice. Both *Sox9* conditional knockdown and control mice display hind limb paralysis immediately following SCI on day 1 post-SCI. *Sox9* conditional knockdown mice score higher (increased hind limb function) on the Basso Mouse Scale (BMS) in comparison to control mice every week between 1 and 14 weeks after SCI (p<0.05, 2 way repeated measures ANOVA, Newman-Keuls post-hoc tests (p<0.05); n=9 SOX9 KO, n= 11 control). B) *Sox9* conditional knockdown mice demonstrate increased distance traveled in comparison to control injured mice measured over a 2 h period in a rodent activity box (p<0.05, 1-way ANOVA; n=9 SOX9 KO, n= 11 controls).
Figure 4. Sox9 conditional knockdown mice demonstrate improved locomotor recovery after SCI.

A

BMS Score vs. Time Post-SCI

- Injured SOX9 KO
- Injured Controls

B

Total Distance Traveled (cm)

- Injured Controls
- Injured SOX9 KO
- Uninjured Wildtype
- Uninjured SOX9 KO
Sox9 conditional knockdown mice display reduced CSPG, collagen and GFAP expression at the lesion site 14 weeks following SCI

Since the Sox9 conditional knockdown animals had reduced levels of XT-I, C4st-1, Age, brevican, neurocan, Col2a1, Col4a1, and GFAP mRNA at 1 week post-SCI and concomitant reductions in CSPG and GFAP protein levels at the lesion 2 weeks post-SCI, we anticipated that these animals would display reduced evidence of a glial scar at 14 weeks post-SCI. Spinal cord sections from Sox9 conditional knockdown mice at 14 weeks after SCI demonstrated a significant reduction in CSPG immunoreactivity (area immunoreactivity per cord area) rostral to, caudal to and at the lesion epicenter compared to controls (Fig. 5). Decreased CSPG staining correlated with increased BMS scores by linear regression analysis ($r^2 = 0.69$). Quantifying the area of positive trichrome staining for collagen (blue stain in Fig. 6) demonstrated reduced amounts of collagen (area immunoreactivity per cord area) in the lesion epicenter in Sox9 conditional knockdown mice compared to controls. Finally, in agreement with the Q-PCR data at 1 week post-SCI and the protein quantitation at 2 weeks post-SCI, immunohistochemistry demonstrated reduced expression of GFAP (area immunoreactivity per cord area) in Sox9 conditional knockdown mice rostral to, caudal to and at the lesion epicenter 14 weeks post-injury (Fig. 7). Together these data indicate that at 14 weeks post-injury, the lesion of spinal cord injured Sox9 conditional knockdown animals contains fewer reactive astrocytes and less glial and collagenous scarring than that of the control animals.
Figure 5. Sox9 conditional knockdown mice display reduced CSPG expression 14 weeks post-SCI. Representative photomicrographs of anti-CSPG DAB immunohistochemical staining approximately 1 mm rostral to the lesion epicenters (A) at the epicenters (B) and 1 mm caudal to the lesion epicenters (C) from Sox9 conditional knockdowns and controls as indicated. D) Quantification of area of CSPG immunoreactivity in Sox9 conditional knockdown and control sections. The area of immunostaining per cross-sectional area of spinal cord was quantified using ImageProPlus software on sections spaced 160 μm apart. The area per area measurements were then grouped into bins centered on the positions indicated. The bin representing epicenter in each animal extended 0.65 mm rostral and caudal to the center of the lesion. The bins rostral and caudal to the epicenter were centered on the positions shown relative to the epicenter and included sections 0.4 mm rostral and caudal. * indicates statistically significantly different from controls (p<0.05, 2 way ANOVA, Newman-Keuls post-hoc test (p<0.05); n = 9 Sox9 KO, n =11 controls). Bars = 100 μm.
Figure 5. Sox9 conditional knockdown mice display reduced CSPG expression 14 weeks post-SCI.
Figure 6. Sox9 conditional knockdown mice demonstrate reduced collagen at the lesion epicenter 14 weeks post-SCI. A) Representative photomicrographs of Trichrome-stained spinal cord sections from the lesion epicenters of Sox9 conditional knockdown and control mice. B) High power magnifications of boxed areas in A). C) Quantification of area of collagen (blue) staining in Sox9 conditional knockdown and control sections. Areas of collagen staining were quantified as explained in legend to Figure 5. * indicates statistically significantly different from controls (p<0.05, 2 way ANOVA, Newman-Keuls post-hoc test (p<0.05); n=9 Sox9 KO, n = 11 control). Bars = 100 µm.
Figure 6. Sox9 conditional knockdown mice demonstrate reduced collagen at the lesion epicenter 14 weeks post-SCI.
Figure 7. *Sox9* conditional knockdown mice demonstrate reduced GFAP expression 14 weeks post-SCI. A) Representative photomicrographs of anti-GFAP immuno-staining from spinal cord sections approximately 1 mm rostral to the lesion epicenters (A) at the epicenters (B) and 1 mm caudal to the lesion epicenters C) from *Sox9* conditional knockdowns and controls as indicated. D) Quantification of area of GFAP immunoreactivity (area per area) in *Sox9* conditional knockdown and control sections. Areas of GFAP immuno-staining were quantified as explained in legend to Figure 5. * indicates statistically significantly different from controls (p<0.05, 2 way ANOVA, Newman-Keuls post-hoc test (p<0.05); n=9 *Sox9* KO, n = 11 controls). Bars = 100 µm.
Figure 7. Sox9 conditional knockdown mice demonstrate reduced GFAP expression 14 weeks post-SCI.
**Sox9 conditional knockdown mice have increased neurofilament-positive fibers in the penumbra of the lesion site following SCI**

As the reduced CSPG and collagen levels in the *Sox9* conditional knockdown lesions would be predicted to correlate with an environment more permissive to axonal growth and sprouting, we expected to observe an increased number of neurofilament-positive fibers in the spinal lesions of the *Sox9* conditional knockdown mice compared to controls. Immuno-stained spinal cord sections from *Sox9* conditional knockdowns and controls demonstrated significant reductions in neurofilament at the lesion epicenter (Fig. 8). Whereas the area of neurofilament immunoreactivity (area immunoreactivity per cord area) in the *Sox9* conditional knockdown mice was not significantly different from controls at the lesion epicenter, neurofilament immunoreactivity was increased in the bins 0.8 mm rostral and caudal to the lesion epicenter in *Sox9* conditional knockdowns compared to controls (Fig. 8).

**Sox9 knockdown mice display increased 5-HT immunoreactivity caudal to the lesion site following SCI**

Descending serotonergic (5-HT positive) projections from the raphe nuclei control a variety of normal body functions. Serotonergic projections synapsing in the dorsal horn modulate pain sensation [34, 35], serotonergic projections targeting sympathetic preganglionic neurons in the intermediolateral cell column (IML) contribute to autonomic regulation [36], and serotonergic projections synapsing in the ventral horn provide excitatory input to motor neurons, the loss of which correlates with locomotor dysfunction [37]. To evaluate whether the improved recovery achieved by the *Sox9* conditional knockdown mice could be attributed to increased
Figure 8. *Sox9* conditional knockdown mice demonstrate increased neurofilament immunoreactivity rostral and caudal to their lesion epicenters 14 weeks post-SCI. A) Representative photomicrographs of anti-neurofilament immuno-staining from spinal cord sections approximately 0.5 mm rostral to the lesion epicenters (A) at the epicenters (B) and 0.5 mm caudal to the lesion epicenters C) from *Sox9* conditional knockdowns and controls as indicated. D) Quantification of area of neurofilament immunoreactivity (area per area) in *Sox9* conditional knockdown and control sections. Areas of neurofilament immuno-staining were quantified as explained in legend to Figure 5. * indicates statistically significantly different from controls (p<0.05, 2 way ANOVA, Newman-Keuls post-hoc test (p<0.05); n=9 *Sox9* KO, n = 11 control). Bars = 100 µm.
Figure 8. Sox9 conditional knockdown mice demonstrate increased neurofilament immunoreactivity rostral and caudal to their lesion epicenters 14 weeks post-SCI.
Figure 9. *Sox9* conditional knockdown mice display increased 5-HT immunoreactivity caudal to the lesion. Immunohistochemistry was used to detect serotonin in the spinal cord 14 weeks post-SCI. A) Representative photomicrographs of sections stained for 5-HT immunoreactivity ~1.2 mm caudal to the lesion epicenter from *Sox9* conditional knockdown and control mice. Almost no 5-HT immunoreactivity was observed caudal to the lesion in control mice, however 5-HT immunoreactivity was observed caudal to the lesion in SOX9 conditional knockdown mice. B) High power magnifications of boxed areas in A). C) Quantification of 5-HT immunoreactivity. 5-HT-immunoreactivity was significantly increased in intermediolateral cell column and the ventral horn of *Sox9* conditional knockdown mice in comparison to control mice. * indicates statistically significantly different from controls (Student’s T-test, p=<0.05; n=9 *Sox9* KO, n = 11 controls). Bars = 100 µm.
Figure 9. Sox9 conditional knockdown mice display increased 5-HT immunoreactivity caudal to the lesion.

A  Control  SOX9 KO

B  Control  SOX9 KO

C  Serotonin (5-HT) staining in the IML caudal to epicenter

D  Serotonin (5-HT) staining in the ventral horn caudal to epicenter
5-HT inputs onto targets caudal to the injury we performed immunostaining for 5-HT on cross-sections from Sox9 conditional knockdowns and controls 14 weeks after SCI. Between 0.8 mm - 1.6 mm caudal to the lesion epicenter Sox9 conditional knockdown mice displayed a statistically significant increase in 5-HT immunoreactivity in the IML and ventral horn, compared to control mice (Fig. 9).

**Sox9 conditional knockdown mice display decreased WFA caudal to the lesion site following SCI**

In addition to their contribution to the glial scar matrix CSPGs are also a major component of the PNN ECM that stabilize synapses during development [38] and limit plasticity in the adult nervous system. Since Sox9 conditional knockdowns demonstrated reduced levels of CSPGs and other ECM components at the glial scar we evaluated whether they may also demonstrate reductions in the ECM in their PNNs. Cross-sections from Sox9 conditional knockdowns and controls were stained with biotinylated Wisteria floribunda agglutinin (WFA). WFA binds N-acetylgalactosamine side chains in proteoglycans including CSPGs [39, 40]. Cross-sections 1.6 – 3.2 mm caudal of the lesion epicenter were selected for WFA-staining to determine whether conditional Sox9 ablation might lead to a reduction in PNN ECM distal to the lesion. WFA staining revealed a reduction in PNNs in the Sox9 knockdown mice caudal to the lesion compared to control mice 14 weeks post-SCI (Fig. 10). This suggests that ablation of Sox9 creates a more growth-permissive environment in the mouse spinal cord, possibly contributing to improved motor function after spinal cord injury.
Figure 10. Perineuronal net matrix is reduced in Sox9 conditional knockdown mice caudal to lesion 14 weeks post-SCI. WFA staining was used to detect PNN matrix caudal to lesion epicenter in control and Sox9 conditional knockdown mice. A) Representative photomicrographs of sections stained for WFA caudal to the lesion epicenter from Sox9 conditional knockdown and control mice. B) High power magnifications of boxed areas in A. C) Quantification of area of WFA staining in Sox9 conditional knockdown and control sections. The area of WFA stained tissue per cross-sectional area of spinal cord was quantified using ImageProPlus software on sections spaced 160 µm apart from 1.6 to 3.2 mm caudal to the epicenters. * indicates statistically significantly different from controls (Student’s T-test, p<=0.05; n=9 Sox9 KO, n = 11 controls). Bars = 100 µm.
Figure 10. Perineuronal net matrix is reduced in Sox9 conditional knockdown mice caudal to lesion 14 weeks post-SCI.
2.4 Discussion

Although the glial scar plays a key role in the acute response to SCI by sealing the lesion site, restoring homeostasis, and modulating immunity, it also presents an obstacle to recovery [41]. In the adult mammalian CNS, glial scarring is a major constraint to successful axonal regeneration. The scar impedes axonal growth through the lesion and leads to either misrouting or growth arrest, both ultimately resulting in chronic denervation [42-45]. These anti-regenerative properties of the glial scar are predominantly caused by the build-up of various proteoglycans and collagens after injury [46-48]. Having previously demonstrated that siRNA knock-down of the transcription factor SOX9 down-regulates the expression of CSPG-synthetic enzymes in astrocyte cultures [27], we investigated the effect of in vivo Sox9 conditional knockdown on gene expression, glial scarring and functional recovery after SCI.

In addition to our siRNA work demonstrating SOX9 regulation of xylosyltransferase-I and II and C4st-1[27], others have shown that SOX9 also regulates the expression of Col2a1 [49, 50], Col4a1 [51], the CSPG core protein Agc [52], and Crtl [53]. 4-hydroxytamoxifen-induced knock-down of SOX9 expression in astrocyte cultures was accompanied by reduced mRNA expression of most of the predicted SOX9 target genes. In the injured spinal cord of tamoxifen-treated Sox9 Δlox/lox,Cre mice the mRNA expression of these same genes with the addition of C4st-1, Col4a1 were also significantly reduced compared to controls; this was paralleled by reductions in SOX9, GFAP and CSPG protein levels. These results largely confirm the anti-Sox9 siRNA results in rat primary astrocytes previously reported [27] and demonstrate that the SOX9 target genes identified in astrocyte cell culture experiments are also regulated by SOX9 in vivo in the injured spinal cord.
Some predicted SOX9 target genes did not show reduced expression in Sox9 knockdown astrocyte cultures or spinal cord-injured mice. XT-II expression was not reduced in either the Sox9\(^{\text{flax/flax;}}\text{Cre}\) astrocyte cultures or spinal cord injured mice, suggesting that SOX9 activity is not necessary for the expression of this isoform of xylosyltransferase. The reduction of XT-II expression that we previously described in rat primary astrocyte cultures may simply reflect a minor species difference in SOX9 activities between rats and mice. Whereas C4st-1 expression was not reduced in Sox9\(^{\text{flax/flax;}}\text{Cre}\) astrocyte cultures it was reduced in the injured spinal cords of Sox9\(^{\text{flax/flax;}}\text{Cre}\) mice. This may reflect in vitro versus in vivo differences in the regulation of C4st-1 gene expression. The greater dependence C4st-1 expression on SOX9 activity in the injured spinal cord may be due to the large increase in SOX9 expression that occurs within the first 12 hours of SCI that may mask the effects of other regulators that are important to C4st-1 expression in vitro. Expression of Col4a1, like C4st-1, was not reduced in the Sox9 conditional knockdown astrocyte cultures but was reduced in the spinal lesions of Sox9 conditional knockdown mice after SCI. Others have shown that collagen 4 is expressed in the lesion epicenter in a directly adjacent but non-overlapping pattern with the GFAP-positive astrocytes in the surrounding penumbra [54]. Together this suggests that SOX9 is required for the expression of Col4a1 in the cells (likely meningeal fibroblasts) that produce collagen 4 in the lesion epicenters. Finally Crtl expression was not reduced in the Sox9 conditional knockdown cultures or in the Sox9 conditional knockdown mice after SCI suggesting that SOX9 activity is not necessary for Crtl expression. This result was surprising as others have demonstrated that anti-Sox9 siRNA transfection into a human chondrosarcoma cell line, OUMS-27, resulted in reduced levels of Crtl expression [53]. However this same group also demonstrated the presence of a SOX9-independent enhancer element in the 5’-UTR of the Crtl gene. Thus we suggest that this
SOX9 independent enhancer element may be regulating expression of *Crtl* in astrocyte cultures and in the injured spinal cord.

The *Sox9* conditional knockdown mice achieved significantly higher BMS scores than controls, improved rates of plantar placement and traversed 2-3 times the distance traversed by controls 14 weeks post-SCI. The strong correlation ($r^2 = 0.61$) between BMS scores at 14 weeks post-SCI and decreased area of CSPG staining at the lesion site suggests that the improved locomotor function in the *Sox9* conditional knockdowns is due to the decreased CSPG expression in these mice following SCI. The reduced expression of *XT-I, C4st-1, Agc*, brevican, neurocan, *Col2a1* and *Col4a1* would be expected to produce a less fibrous scar with less CSPG content. Gomori’s trichrome staining 14 weeks post-injury confirmed that the *Sox9* conditional knockdown lesions had less collagen than controls. Immunohistochemistry further demonstrated that there was less CSPG content in the *Sox9* conditional knockdown lesions than controls. Neurofilament immuno-staining showed that the area of neurofilament immunoreactivity at the lesion epicenter did not differ significantly between *Sox9* conditional knockdowns and controls. This suggests that the improved locomotor recovery in *Sox9* conditional knockdowns is not due to a greater degree of axonal sparing in these mice nor is it likely due to greater numbers of axons traversing the lesion site to connect caudal to the lesion. However neurofilament immuno-staining rostral and caudal to the lesion epicenters was increased in the *Sox9* conditional knockdown mice, consistent with the suggestion that the reduced CSPG content in the penumbra of the *Sox9* conditional knockdowns permits greater amounts of axonal sprouting. Axonal sprouting rostral and caudal to the lesion may allow the formation of connections with spared propriospinal neurons and underlie the improved locomotor behavior in the *Sox9* conditional knockdowns.
CSPG immunoreactivity and WFA-staining demonstrate reduced CSPG expression and PNN matrix rostral and caudal to the penumbra 14 weeks post-SCI in the Sox9 conditional knockdowns. Evidence suggests that CSPGs are up-regulated within PNNs both near and far from a CNS lesion [55]. PNNs are produced by both neurons and astrocytes and surround synapses throughout the nervous system [39]. CSPG expression in PNNs is low during the initial stages of synaptogenesis, and dramatically increases toward the end of developmental plasticity [56, 57]. Enzymatic digestion of PNNs by chondroitinase ABC leads to reactivation of plasticity in adult animals [57, 58], and enhances spared fiber collateral sprouting and synapse formation in injured animals [59]. Thus reduced SOX9 expression may open a window for increased plasticity following SCI at denervated sites within and remote to the lesion. In the Sox9 conditional knockdowns, reduced PNNs and CSPG content in the PNNs may have permitted spared propriospinal or descending supraspinal axons to synapse on deafferented targets caudal to the lesion. Evidence in support of this comes from the 5-HT immuno-staining which clearly showed increased 5-HT immunoreactivity caudal to the lesion around the IML and ventral horn in Sox9 conditional knockdowns.

The postulate that reduced CSPGs in the PNNs distal to the lesion may account for the improved locomotor recovery observed in the Sox9 conditional knockdowns might explain other facets of the recovery in these mice. For example the Sox9 conditional knockdowns have higher BMS scores than controls starting at one week post-SCI. This improvement in locomotor activity seems to occur too quickly to be explained by long-range regeneration of inputs rostral to the lesion. Furthermore the absence of increased neurofilament immunoreactivity at the lesion epicenter also argues against any significant amount of regeneration across the lesion epicenter. However short-range sprouting of spared axons onto deafferented targets promoted by reduced
CSPGs in PNNs would be expected to occur rapidly. This type of repair may also be less prone to mis-wiring of circuits as the most likely axons to form new synapses on a target will be those that are already innervating adjacent neurons in the same field. This type of repair may be far less demanding on axonal growth and targeting than long range regeneration.

A second possible explanation for improved locomotor recovery of the Sox9 conditional knockdowns may be the reduced GFAP expression in these mice after SCI. Unlike the other SOX9 target genes that are involved in ECM production, GFAP is a cytoskeletal protein and used most often as a marker of astrocyte activation [60]. Its identification as part of a battery of genes up-regulated by SOX9 after SCI suggests that SOX9 not only up-regulates the expression of ECM-related genes but also regulates the overall state of astrocyte activation and response to injury. In addition to being a major producer of the glial scar, astrocytes also play an important role in inflammation. In response to TGF-β1 astrocytes up-regulate expression of the pro-inflammatory genes nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2) [61]. IL-1β and TNF-α also increase astrocyte production of nitric oxide [61, 62]. In the Sox9 conditional knockdown mice, reduced GFAP expression might indicate less astrocyte activation and perhaps less production of pro-inflammatory mediators. Thus a muted inflammatory response may account for some of the improved recovery observed in the Sox9 conditional knockdowns.

A third possible explanation for improved locomotor recovery of the Sox9 conditional knockdowns rests on evidence that SOX9 may play a role in neural stem differentiation. Expression studies have shown that SOX9 is expressed by neuroepithelial cells in the ventricular zone of the developing spinal cord, by oligodendrocytes and by astrocytes but not by neurons [63]. Knocking out Sox9 in the developing mouse spinal cord results in perinatal lethality,
decreased numbers of oligodendrocyte progenitors and astrocytes and an increased number of motor neurons [63] and neuroblasts [64]. MicroRNA studies also suggest that SOX9 is gliogenic, promoting neural stem cells to adopt an astrocyte or oligodendrocyte fate [65]. Following injury, neural stem cells proliferate and differentiate almost exclusively into astrocytes [66, 67] that generate scar, but not into neurons [66]. These results are consistent with the hypothesis that, in neural stem cells, SOX9 expression promotes a glial rather than a neuronal cell fate and that, in Sox9 conditional knockdowns, neural stem cells activated by the injury may adopt a neuronal as opposed to a glial fate. If astrocytes newly-born after injury contribute to CSPG production, or if newly-born neuroblasts are able to generate new neurons or produce growth factors that support neuronal survival [68-70], then the effect of Sox9 ablation on neural stem cell behavior (decreasing the number of newborn astrocytes and increasing the number of neuroblasts or neurons) may explain the improved recovery of Sox9 conditional knockdown mice after SCI. This possibility is being evaluated by fate mapping studies in the Sox9 conditional knockdowns after SCI.

In summary, Sox9 conditional knockdown improved hind limb motor function in mice following T9 SCI. The improved recovery in the Sox9 conditional knockdowns correlated with reduced GFAP, collagen, and CSPG expression at the lesion and at sites distant from the lesion. We suggest that the reduced CSPG expression at the glial scar and in PNNs distant to the injury opened up a window of opportunity for increased local plasticity possibly allowing for the formation of new propriospinal connections or sprouting from spared axons onto deafferented targets below the lesion. This explanation is consistent with the neurofilament immunoreactivity showing increased fiber sprouting in the lesion penumbra but no increase in fibers that traverse the lesion epicenter. It is also consistent with the demonstrated reduction in PNNs caudal to the
lesion and increased 5-HT immunoreactivity demonstrating more serotonergic inputs onto IML and ventral horn targets caudal to the lesion. These results suggest that inhibition of SOX9 activity may be a novel therapeutic strategy for the treatment of SCI.

2.5 Acknowledgments

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2.6 Author Disclosure Statement

A.B. holds a patent on SOX9 inhibition as a target for regeneration in the nervous system. No competing financial interests exist for W.M.M.
2.7 Supplementary Table 1. Two Way ANOVA Summary Table

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2.8 References


Chapter 3: Conditional ablation of Sox9 after spinal cord injury reduces chondroitin sulfate proteoglycan expression and improves locomotor recovery

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Running title: Delayed SOX9 knockdown after SCI improves locomotor recovery

Keywords: SOX9; spinal cord injury; CSPG; reactive astrocytes; glial scar; perineuronal nets
3.0 Abstract

Chondroitin sulfate proteoglycans (CSPGs) limit neuroplasticity during development and following spinal cord injury (SCI). We have previously identified SOX9 as a transcription factor that up-regulates the expression of CSPGs and have demonstrated that Sox9 ablation prior to SCI leads to reduced CSPG expression and improved locomotor recovery. The present study sought to determine whether Sox9 ablation would reduce CSPG levels in the injured cord and improve locomotor recovery if initiated a week after injury. Sox9^fl/fl^ (control) and Sox9^fl/fl;Cre^ (Sox9 knockdown) mice were subjected to a 70 kdyne contusion SCI at thoracic spinal cord level 9. One week after injury, tamoxifen was administered to ablate Sox9. Quantitative-PCR demonstrated that this experimental protocol caused Sox9 mRNA levels to decline at 13 days post-SCI. Six weeks post-SCI mice with delayed Sox9 knockdown expressed reduced levels of CSPG core proteins (neurocan and aggrecan), and glial fibrillary acidic protein (a marker of astrocyte activation) in the injured spinal cord compared to controls. These changes in gene expression were accompanied by improved hind limb function and locomotor recovery as evaluated by the Basso Mouse Scale (BMS) and rodent activity boxes. Histological assessments confirmed reduced CSPG deposition at the lesion site and in perineuronal nets of mice with delayed Sox9 knockdown, and demonstrated increased serotonin immunoreactivity caudal to the injury site. Improved recovery following Sox9 knockdown delayed for 1 week after SCI highlights the clinical potential of an anti-SOX9 treatment for SCI.
3.1 Introduction

The limited spontaneous recovery from spinal cord injury (SCI) observed in humans [1, 2] and adult animals [3, 4] has been attributed, in part, to injury-induced neuroplasticity. Axon growth that underlies structural neuroplasticity has been shown to be limited by chondroitin sulfate proteoglycans (CSPGs) \textit{in vitro} [5-7] and \textit{in vivo} [8-13]. CSPGs are a family of proteins composed of a core protein with chondroitin sulfate side chains [14]. In the injured spinal cord CSPGs are a major component of the glial scar that is mostly produced by reactive astrocytes [15, 16], with contributions from macrophages, microglia, oligodendrocytes, invading Schwann cells and meningeal fibroblasts [17]. Enzymatic removal of chondroitin sulfate side chains from CSPGs at the lesion site using chondroitinase ABC has been demonstrated to increase axon sprouting and neurological recovery [8, 9]. CSPGs are also found in extracellular matrix structures called perineuronal nets (PNNs) that surround the cell bodies and dendrites of some classes of neurons [18]. CSPGs in PNNs have been suggested to stabilize synapses by preventing axonal sprouting onto inappropriate targets after appropriate connections have been made during development [11]. Enzymatic digestion of PNNs by chondroitinase leads to reactivation of plasticity in adult animals [19, 20].

For several years our laboratory has been studying the molecular mechanisms that control CSPG production by reactive astrocytes with the goal of developing a strategy to reduce CSPG expression after SCI. This work led us to the identification of SOX9 as a transcription factor that up-regulates the expression of CSPGs in primary astrocytes [21]. Using a line of tamoxifen-inducible conditional \textit{Sox}9 knockdown mice, we subsequently demonstrated that SOX9 regulates the expression of a battery of genes involved in astrocyte activation and CSPG production and that SOX9 ablation reduces CSPG levels at the lesion site and in PNNs distant to the SCI [22].
We also demonstrated that conditional SOX9 ablation leads to improved locomotor outcomes after SCI. One of the difficulties in this previous study was that we elected to ablate Sox9 before carrying out the SCI. Tamoxifen was administered once per day for one week, two weeks prior to SCI in order to allow one week for the Cre-mediated recombination to occur and one week for tamoxifen wash-out. This experimental design allowed us to maximize the effects of SOX9 ablation on scar formation but did not answer the more clinically relevant question - whether SOX9 ablation after scar formation has already begun could appreciably reduce CSPG levels in the injured cord and improve neurological outcomes. In the present study we report that ablating Sox9 during the second week after SCI results in reduced levels of CSPGs at the lesion site and in PNNs in the lumbar enlargement. These reductions in CSPGs are accompanied by improved locomotor recovery in the spinal cord-injured Sox9 conditional knockdown mice.

3.2 Materials and Methods

**Sox9 conditional knockdown mice**

Mice carrying floxed Sox9 (exons 2 and 3 of Sox9 surrounded by loxP sites) alleles [23] (Sox9\(^{\text{flox/flox}}\)) were crossed with a transgenic mouse line that expresses Cre recombinase fused to the mutated ligand binding domain of the human estrogen receptor (ER) under the control of a chimeric cytomegalovirus immediate-early enhancer/chicken β–actin promoter (B6.Cg-Tg(CAG-Cre/Esr1)5Amc/J)[24] (Jackson Laboratories, Bar Harbor, Maine). The mutated ER ligand binding domain of the fusion protein binds tamoxifen [25]. The Cre-ER fusion protein remains trapped in the cytoplasm of all cells due to interactions with Hsp90. Tamoxifen binds to the mutated ER portion of the fusion protein releasing it from Hsp90 allowing its transport to the
nucleus where it excises \( \text{loxP} \)-flanked \( \text{Sox9} \) DNA [24]. Tamoxifen-treated \( \text{Sox9}^{\text{flox/flox}};\text{CAGGCreER} \) \( \text{Sox9}^{\text{flox/flox}};\text{Cre} \) mice served as inducible \( \text{Sox9} \) knockdown animals and their tamoxifen-treated \( \text{Sox9}^{\text{flox/flox}} \) littermates (not carrying the Cre transgene) served as control animals (expressing normal levels of SOX9). Tamoxifen (Sigma Aldrich, St. Louis, Missouri) was administered at 3 mg/20 g mouse by oral gavage to all \( \text{Sox9}^{\text{flox/flox}};\text{Cre} \) and \( \text{Sox9}^{\text{flox/flox}} \) littermates starting 7 days post-SCI, once per day, for 7 days.

Animals were genotyped by polymerase chain reaction (PCR) analysis using the following primers:

\( \text{Sox9}^{\text{flox}} \) allele: 5′-ACACAGCATAGGCTACCTG-3’ and 5′-TGGTAATGAGTCATACACGTAC-3’.

\( \text{Sox9}^{\text{wildtype}} \) allele: 5′-GGGGCTTGTCTCCTTCAGAG-3’ and 5′-TGGTAATGAGTCATACACGTAC-3’.

\( \text{Sox9}^{\text{knockdown}} \) allele: 5′-GTCAAGCGACCCATG-3’ and 5′-TGGTAATGAGTCATACACGTAC-3’.

\( \text{Cre}^+ \) allele: 5′-CAATTTACTGACCGTAC-3’ and 5′-AGCTGGCCCAAATGTTGCTG-3’.

**Spinal cord injury**

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the Guide to Care and
Use of Experimental Animals prepared by the Canadian Council on Animal Care. $^{Sox9\text{flo}x/flo}{\text{x}};{\text{Cre}}$ and $^{Sox9\text{flo}x/flo}$ mice were anesthetized with 100 mg/kg ketamine: 5 mg/kg xylazine. The spinal cord was stabilized at vertebra T7 and T9 with forceps, and a T8 dorsal laminectomy was performed to expose spinal segment T9. The Infinite Horizons Impactor was used to deliver a 70 kdyne contusion injury with a 1 s dwell time (displacement range: 500-900 $\mu$M) to spinal segment T9 (Precision Systems and Instrumentation, Fairfax, Virginia). Following SCI the mice were housed individually. Baytril (25 mg/kg, Bayer, Toronto, Ontario, Canada) and buprenorphine (0.01 mg/kg, Schering-Plough, Hertfordshire, UK) were injected subcutaneously twice daily for 3 days post-SCI. Bladders were emptied manually twice daily for the duration of the experiment. The experimental timeline is shown in Figure 1.

**Quantitative-PCR**

RNA was extracted from a 5 mm segment of spinal cord centered on the lesion of delayed $^{Sox9}$ knockdown and control mice 6 weeks post-SCI, using the RNA-Easy kit according to the manufacturer's instructions (Qiagen, Valencia, California). First strand cDNA was synthesized from 1 $\mu$g RNA per sample using the High Capacity cDNA Archive Kit according to the manufacturer instructions (Applied Biosystems, Carlsbad, California). The primer probe sets, optical adhesive covers, and quantitative-PCR (q-PCR) plates were purchased from Applied Biosystems. All primer probes were labeled with 5'TAMRA as quencher. TaqMan assays were conducted using the Applied Biosystems gene expression assay primer probe sets listed in Table 1.
Figure 1. Experimental timeline. Sox9\textsuperscript{flox/flox}/Cre and Sox9\textsuperscript{flox/flox} control mice were subjected to a SCI at T9 using the infinite Horizons Impactor. Beginning at 7 days post-SCI all mice received daily administrations of tamoxifen for one week. Changes in Sox9 mRNA levels at the lesion were monitored by Q-PCR in a subset of animals on days 2, 4 and 6 after initiating tamoxifen administration (corresponding to days 9, 11 and 13 after SCI). Changes in SOX9 target gene protein and mRNA expression levels at the lesion by Western blot analyses and Q-PCR was carried out 6 weeks post-SCI. Hind limb function was assessed using the BMS on the day following SCI and then at weekly intervals until 14 weeks post-SCI. Locomotor activity was assessed using rodent activity boxes at 1 week, 6 weeks and 14 weeks post-SCI. After sacrificing the mice at 14 weeks post-SCI their spinal cords were removed for immunohistochemical analyses.
Figure 1. Experimental timeline.

SCI
(n=31 Controls, n=33 KO)

WEEK

Tamoxifen

Sox9 Knockout mRNA timecourse
(n=3 per timepoint):

Western blot and mRNA analyses
(Western n=4, Q-PCR n=5):

Basso Mouse Scale hind limb function testing
(n=11 Controls, 12 KO):

Activity Box testing
(n=11 Controls, 12 KO):

Immunohistochemistry
(n=8 Controls, 9 KO):
Table 1. List of TaqMan Real-Time PCR Primer Probe Sets

<table>
<thead>
<tr>
<th>Primer Probe</th>
<th>Catalog Number</th>
<th>PCR Ct Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Mm99999915_g1</td>
<td>17.04-18.65</td>
</tr>
<tr>
<td>Sox9</td>
<td>Mm00448840_m1</td>
<td>22.10-25.97</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Mm00545807_m1</td>
<td>23.47-25.97</td>
</tr>
<tr>
<td>Neurocan</td>
<td>Mm00484007_m1</td>
<td>23.48-29.98</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mm01253033_m1</td>
<td>17.04-18.97</td>
</tr>
</tbody>
</table>

TaqMan (Applied Biosystems) gene expression assays were conducted on a 7900HT fast q-PCR apparatus (Applied Biosystems) using thermal cycler conditions set as follows; 10 min at 95°C followed by 40 cycles of 30 s at 95°C followed by 30 s at 60°C. Cycle thresholds (Ct) for all target genes were kept below 30 as indicated in Table 1. Target gene mRNA expression was normalized to the amount of GAPDH mRNA present in each sample and analyzed using the comparative Ct method [26]. The ratio of knockdown to control sample normalized target gene mRNA was analyzed by Student's T-test.

Time course of Sox9 expression after tamoxifen administration

Sox9$^{flax/flax, Cre}$ and Sox9$^{flax/flax}$ control mice underwent SCI as described above. Tamoxifen administration began 1 week after SCI. Mice were sacrificed at 2, 4, and 6 days after
the start of tamoxifen administration and Q-PCR was carried out to characterize the time course with which Sox9 mRNA levels decline following tamoxifen administration in Sox9^flox/flox;Cre mice.

**Western blotting**

Protein was isolated from a 5 mm segment of spinal cord tissue centered on the lesion site in delayed Sox9 knockdown and control mice 6 weeks post-SCI. The spinal cord tissue was lysed in modified RIPA buffer (1% nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA, pH 7.5, plus 1 complete Mini Protease Inhibitor tablet/7mL RIPA buffer (Roche Molecular Biochemicals, Indianapolis, Indiana) on ice using a ground glass homogenizer. The protein mixture was centrifuged at 13,000 x g for 5 min and the supernatant collected. For the neurocan and aggrecan analyses the protein aliquots (at a concentration of 2–3 mg/ml) were treated with 0.3 U/ml chondroitinase ABC (Sigma-Aldrich) for 8 h at 37 °C. The protein samples were diluted in reducing PAGE loading buffer, and loaded on reducing SDS-PAGE gels at 10 μg/well. The membrane was blocked in 10% nonfat powdered milk and then incubated with primary antibodies; anti-SOX9 (AB 5535, Millipore, Billerica, Massachusetts used at 1:1000), anti-glial fibrillary acidic protein (GFAP; MAB360, Millipore, Billerica, Massachusetts used at 1:1000), anti-neurocan (MAB5212, Millipore, Billerica, Massachusetts used at 1:1000), anti-aggrecan (WH0000176M1, Sigma, St. Louis, Missouri, used at 1:1000), and anti-β-actin (A1978, Sigma, St. Louis, Missouri, used at 1:10,000) for protein expression assessed by western blot. Horse radish peroxidase (HRP)-conjugated anti-mouse IgG (715-035-151, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and HRP conjugated
anti-rabbit IgG (711-035-152, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) secondary antibodies were used at 1:20,000 dilution to detect protein expression. SOX9, GFAP, neurocan, and aggregcan protein expression was normalized to β-actin protein expression by densitometry using the EpiChemi Darkroom (UVP Bioimaging Systems, Upland, California) and LabWorks software (Media Cybernetics Inc, Bethesda, Maryland).

**Behavioral testing**

All aspects of the behavioral testing and data analyses completed in this study were done using a blinded experimental design. Locomotor recovery of the mice was assessed by two observers, blinded to animal genotypes, using the Basso Mouse Scale (BMS) open field locomotor score [27]. The day following SCI, all mice were evaluated for any signs of locomotor recovery in their hindlimbs, and mice that had BMS scores > 0.5 were excluded from further analyses (3 delayed Sox9 knockdowns and 2 controls). Animals were evaluated once per week for 14 weeks after SCI. Left and right hind limb scores were averaged to generate a composite score. In addition, locomotion was evaluated using rodent activity boxes (Accuscan Instruments Inc, Columbus, Ohio). The activity boxes use infrared sensors to track the animal’s movements. The total distance the mice traveled was measured over a 2 h period at night (during their normal awake circadian cycle) at 1, 6, and 14 weeks post-SCI.
Spinal cord sectioning

Fourteen weeks post-SCI the animals were deeply anesthetized with 50 mg/kg ketamine: 5 mg/kg xylazine, and cardiac perfusion was carried out with 20 ml of saline at pH 7.4 followed by 20 ml 4% paraformaldehyde (4% PFA in 0.1 M phosphate buffer at pH 7.4). Spinal cords were dissected and post-fixed for 2 h in 4% PFA followed by cryoprotection in 20% sucrose in 0.1 M phosphate buffer at pH 7.4 at 4 °C overnight. Spinal cords were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A. Inc, Torrance, California), frozen over dry ice, and stored at -80 °C overnight. Frozen cords were then sectioned at 16 µm using a cryostat, and serially thaw-mounted on Superfrost™ glass slides (Fisher Scientific Company, Ottawa, Canada).

Immunohistochemistry

Immunohistochemistry was conducted using the primary antibodies listed in Table 2. Slides were rinsed in PBS and treated with 5% normal goat serum and 0.1% triton-X-100 in phosphate buffered saline (PBS) at room temperature for 1 h and then incubated with the primary antibodies in a humidified chamber at 4 °C overnight. Sections were immunostained for CSPG expression using the monoclonal antibody CS56 that recognizes the terminal portions of chondroitin sulfate-4 or -6 side chains and thus detects a variety of CSPGs [28]. CS56 was detected with a biotinylated goat anti-mouse IgM (Vector laboratories, Burlingame, California) secondary antibody (1:200). Sections were then incubated for 45 min with avidin-peroxidase conjugate (Elite Kit, Vector laboratories, Burlingame, California) at room temperature, and the signal visualized by peroxidase diaminobenzine (DAB, Invitrogen, Carlsbad, California). All
DAB staining was conducted with a 2-min DAB reagent incubation time for spinal cord sections from all delayed Sox9 knockdown and control mice, and were processed at the same time. Sections to be stained for perineuronal nets were washed in PBS 3 x 10 min, and incubated with biotinylated Wisteria Floribunda agglutinin (WFA, Sigma Aldrich, St. Louis, Missouri) (1:1000) for 1 h at room temperature. Sections were then incubated for 45 min with streptavidin conjugated Alexa-Fluor 594 (1:500 Invitrogen, Carlsbad, California) at room temperature, and counter-stained with a fluorescent Nissl stain, N-21479 (Invitrogen) (1:100) for 1 h at room temperature. Sections were stained for serotonin with an antibody against 5-hydroxytryptamine (5-HT) and for GFAP. Anti-5-HT and anti-GFAP antibodies were detected by Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:500, Invitrogen, Carlsbad, California), or Alexa-Fluor 488-conjugated goat anti-mouse IgG (1:500, Invitrogen, Carlsbad, California), for 1 h at room temperature. Slides were then washed in PBS and coverslips applied with ProLong Gold Anti-Fade mounting medium (Invitrogen, Carlsbad, California).

Table 2. List of primary antibodies and stains used for spinal cord staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFAP</td>
<td>1:500</td>
<td>Mouse IgG</td>
<td>Millipore, Billerica, Massachusetts</td>
</tr>
<tr>
<td>Anti-CS56</td>
<td>1:300</td>
<td>Mouse IgM</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
</tr>
<tr>
<td>Anti-5HT</td>
<td>1:500</td>
<td>Rabbit IgG</td>
<td>ImmunoStar, Hudson, Wisconsin</td>
</tr>
<tr>
<td>WFA</td>
<td>1:1000</td>
<td></td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
</tr>
<tr>
<td>Nissl</td>
<td>1:100</td>
<td></td>
<td>Invitrogen, Carlsbad, California</td>
</tr>
</tbody>
</table>
Quantification of GFAP, CS56, 5-HT, and WFA staining

For GFAP and CS56 immunostaining, six 16 μm thick longitudinal sections, 160 μm apart were analyzed using ImagePro Plus software (Media Cybernetics Inc, Bethesda, Maryland). A threshold was set for each immunostain that identified positive signal (staining above background levels). For GFAP and CS56 immunoreactivity the area of positive staining was quantified within an area of interest that was centered on, and spanned 3.5 mm rostral and caudal to the lesion epicenter. This area of interest was kept constant for all sections. The area of 5-HT immunoreactivity (area per area of interest) was quantified in the ventral horns in 16 μm thick cross-sections 160 μm apart obtained 0.8-1.6 mm caudal to the injury site. A single pre-set area of interest was used to define all ventral horn regions in all cords in both the Sox9 knockdown and control animal sections. The area of 5-HT staining was quantified using ImagePro Plus Software (Media Cybernetics Inc, Bethesda, Maryland) using a threshold which identified positive signal (staining above background levels). The area of WFA immunoreactivity in the lumbar enlargement was analyzed using 16 μm thick cross-sections 160 μm apart sampled at the L2 spinal level. A single pre-set area of interest was used to define all ventral horn regions in all cords across sections from both Sox9 knockdown and control animals. Positive WFA staining was quantified using ImagePro Plus Software (Media Cybernetics Inc, Bethesda, Maryland) using a threshold which identified positive signal (staining above background levels).
Statistical analysis

Mean values are expressed ± SE. mRNA levels were subjected to statistical analyses using Student’s T-test. GFAP, CS56, 5-HT and WFA levels were compared between delayed knockdown mice and controls using a Student’s T-test. BMS and activity box results were subjected to statistical analysis using a two-way repeated measures ANOVA followed by a Neuman-Keuls post-hoc test when a significant interaction term was achieved. Analyses were conducted with GraphPad Prism software (GraphPad Software Inc, La Jolla, California), except for two-way ANOVAs which were conducted with SigmaStat software (Systat Software Inc, San Jose, California). Statistical significance was accepted at p<0.05. A two way ANOVA summary table is provided as Supplementary Table 1.

3.3 Results

Tamoxifen administration requires 6 days to knock down Sox9 mRNA levels

In this study we initiated delayed Sox9 ablation by administering tamoxifen for a period of 7 days starting 1 week after SCI. A time course study was carried out to determine how rapidly Sox9 mRNA levels might decline following tamoxifen administration. Sox9 knockdown and control mice were sacrificed at 2, 4 and 6 days after the first tamoxifen administration (corresponding to days 9, 11 and 13 after SCI, respectively) and RNA from a 5 mm segment of their spinal cords centered on the lesion epicenter was isolated and evaluated for SOX9 mRNA levels by Q-PCR. Sox9 knockdown and control mice displayed similar Sox9 mRNA levels until the 6th day after the first tamoxifen administration (13 days after SCI) when Sox9 mRNA expression was significantly reduced in Sox9 knockdown mice compared to controls (Fig. 2).
Figure 2. Tamoxifen administration 1 week post-SCI requires 6 days to achieve significant Sox9 knockdown. Spinal cord-injured Sox9<sup>fl/fl</sup> and Sox9<sup>fl/fl</sup>;Cre mice were administered tamoxifen daily for one week beginning at 7 days post-injury. Sox9<sup>fl/fl</sup> and Sox9<sup>fl/fl</sup>;Cre mice were sacrificed at 2, 4 and 6 days after initiating the tamoxifen administration and RNA from a 5 mm segment of spinal cord centered on the lesion was analyzed for Sox9 mRNA levels by Q-PCR. A decrease in Sox9 mRNA levels is not observed until 6 days after the first tamoxifen dose (13 days after SCI). Values are means +/- S.E. * significantly different from control at the same time point (p <0.05, two-tailed Student t-test, n=3/group).
Sox9 mRNA expression following the onset of tamoxifen administration post-SCI

<table>
<thead>
<tr>
<th>9 Days Post-SCI (2 days of tamoxifen administration)</th>
<th>11 days Post-SCI (4 days of tamoxifen administration)</th>
<th>13 days Post-SCI (6 days of tamoxifen administration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>KO</td>
<td>Control</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Indicates significant difference from control.
Delayed Sox9 ablation results in reduced scar gene expression

We previously demonstrated that Sox9 ablation prior to SCI causes a decrease in the mRNA and protein levels for a variety of genes involved in CSPG biosynthesis and astrocyte activation when measured 1 week after injury [22]. To evaluate whether Sox9 ablation in the second week after SCI would yield similar reductions in SOX9 target gene expression, a subset of spinal cord-injured control and Sox9 knockdown mice were sacrificed at 6 weeks post-SCI. From these mice, 5 mm spinal cord segments centered on the lesions were harvested for mRNA and protein expression analyses. Six weeks after SCI (5 weeks after the first dose of tamoxifen) Sox9 knockdown mice displayed a 72% reduction in Sox9 mRNA, a 25% reduction in GFAP mRNA, a 36% reduction in neurocan mRNA and a 33% reduction in aggrecan mRNA levels (Fig. 3). To determine if the observed reductions in mRNA expression were accompanied by parallel reductions in protein levels, we investigated SOX9, GFAP, neurocan, and aggrecan protein levels by western blot analysis 6 weeks post-SCI. The spinal lesions of Sox9 knockdown mice displayed a 72% reduction in Sox9 protein, a 33% reduction in GFAP protein, a 51% reduction in neurocan protein and an 83% reduction in aggrecan protein (Fig. 3).

Delayed Sox9 knockdown mice show improved hind limb function

We have previously shown that knocking out Sox9 in an adult mouse prior to SCI results in improved hind limb recovery [22]. To investigate whether SOX9 ablation beginning 1 week after SCI also leads to improved hind limb locomotor function after SCI, we evaluated the delayed Sox9 knockdown and control mice weekly in an open field test using the Basso mouse scale (BMS) scoring [29] for locomotor activity for 14 weeks after SCI. Immediately after injury, all mice showed complete or near complete paralysis of the hind limbs, represented by a
Figure 3. *Sox9, GFAP, neurocan and aggrecan* mRNA and protein expression levels are reduced 6 weeks post-SCI following Sox9 ablation initiated at 1 week after injury. Spinal cord-injured *Sox9^{floxflox}* and *Sox9^{floxflox};Cre* mice were administered tamoxifen daily for one week beginning at 7 days post-injury. *Sox9^{floxflox}* and *Sox9^{floxflox};Cre* mice were sacrificed at 6 weeks post-SCI and either protein (n=4/group) or RNA (n=5 per group) was isolated from a 5 mm segment of spinal cord centered on the lesion epicenter. A) SOX9 protein levels (as measured by Western blot analyses) and mRNA levels (as measured by Q-PCR) are reduced in the *Sox9* knockdown mice (p<0.05, Student's t-test). The *Sox9* knockdown mice demonstrate similar reduction in the protein and mRNA levels of GFAP (B), neurocan (C) and aggrecan (D).
Figure 3. Sox9 was increased 6 weeks post-SCI follow after reca ablation mRNA and protein expression later injury.

<table>
<thead>
<tr>
<th>Delayed Sox9</th>
<th>Sox9</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>Knockout</td>
</tr>
<tr>
<td>GFAP</td>
<td>GFAP</td>
<td>β-actin</td>
</tr>
<tr>
<td>Neurocan</td>
<td>Neurocan</td>
<td>β-actin</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Aggrecan</td>
<td>β-actin</td>
</tr>
</tbody>
</table>
score of 0, and on day one following SCI all mice displayed a score of < 0.5. Whereas hind limb function in both delayed Sox9 knockdown mice and controls gradually improved over time the delayed Sox9 knockdowns demonstrated a statistically significant improvement over their control littermates. Locomotor BMS scoring in control mice reached a plateau of 0.63 ± 0.15 at 4 weeks post-SCI, (Fig. 4A). The median BMS score in this group of 0.5 indicates slight (less than 90˚) movement in only one of the two hindlimb ankles. In contrast, the BMS scores of the delayed Sox9 knockdown mice continued to improve past 4 weeks and did not reach a plateau until 10 weeks post-SCI, achieving an average BMS score of 1.48 ± 0.21. The median BMS score for delayed Sox9 knockdown mice of 1.5 indicates an extensive (greater than 90˚) movement in one ankle, and a slight (less than 90˚) movement in the other ankle.

**Sox9 knockdown mice display improved locomotor activity**

As a second measure of locomotor recovery we evaluated the delayed Sox9 knockdown and control mice for their overall level of mobility by tracking their locomotion over a 2-h time period using rodent activity boxes. The total distance traversed in a 2-h period was recorded for each mouse during their normal wake period (at night) on 2 consecutive nights and averaged. One week after SCI, and before tamoxifen administration, the Sox9\(^{\text{flox/flox}}\) control mice (Cre-negative) traversed an average distance of 1412 ± 191 cm in 2 h (Fig. 4B), which was not significantly different from the distance traversed by the Sox9\(^{\text{flox/flox};\text{Cre}}\) mice (1456 ± 252 cm in 2 h). At 6 weeks post-SCI (5 weeks after the first tamoxifen administration) no significant differences in total distance traversed between control mice that traversed an average distance of 1393 ± 235 cm in 2 h, and delayed Sox9 knockdown mice that averaged 1906 ± 451 cm in 2 h.
Figure 4. Sox9 knockdown mice demonstrate improved locomotor recovery compared to control mice. A) Both delayed Sox9 knockdown and control mice display hind limb paralysis immediately following SCI, and on day 1 post-SCI (first time point before week 1). Sox9 knockdown mice score higher (increased hind limb function) on the Basso Mouse Scale (BMS) beginning on the sixth week post-SCI (one week after first tamoxifen administration) in comparison to control mice as determined by a 2-way repeated measures ANOVA followed by a Neuman-Keuls post-hoc test (p<0.05; n=12 Sox9 knockdown mice and n=11 controls). B) Sox9 conditional knockdown mice demonstrate increased locomotion in comparison to control mice. Over a 2 h period in a rodent activity box Sox9 knockdown mice demonstrate increased locomotion in comparison to littermate controls at 14 weeks post-SCI as determined by a 2-way repeated measures ANOVA followed by a Neuman-Keuls post-hoc test (p<0.05; n=12 Sox9 knockdown mice and n=11 controls).
Figure 4. Sox9 knockdown mice demonstrate improved locomotor recovery compared to control mice.

Panel A: BMS Score over time post-SCI for Control and Delayed Sox9 Knockout mice.

Panel B: Total Distance Traveled (cm) for Control and KO mice at 1 Week, 6 Weeks, and 14 Weeks post-SCI.
At 14 weeks after SCI, control mice traversed an average distance of 1731 ± 300 cm in 2 h, whereas the delayed Sox9 knockdown mice traversed an average distance of 3537 ± 301 cm in 2 h. The distance traversed by the injured delayed Sox9 knockdown mice was significantly greater than that traversed by the injured control animals and was not significantly different from tamoxifen-treated uninjured Sox9\textsuperscript{lox/lox};Cre mice (3446 ± 543 cm in 2 h, n=4) or uninjured control mice (3287 ± 396 cm in 2 h, n=4).

**Delayed Sox9 knockdown mice demonstrate decreased CSPG, GFAP and PNN protein expression 14 weeks following SCI**

To investigate CSPG and GFAP expression at the lesion, immunohistochemistry was carried out on spinal cords harvested from Sox9 knockdown and control mice at the end of behavioral testing, 14 weeks post-SCI. Spinal cord sections from delayed Sox9 knockdown mice at 14 weeks after SCI had significant reductions in CSPG (Fig. 5) and GFAP (Fig. 6) immunoreactivity (area immunoreactivity per area of interest). In addition to their contribution to the glial scar matrix, CSPGs are also a major component of the PNN that stabilizes synapses during development [11] and limits plasticity in the adult nervous system. Since delayed Sox9 knockdowns had reduced levels of CSPGs at the lesion site we evaluated whether they may also have reductions in their PNNs distant to the lesion. Cross-sections from the lumbar enlargement of delayed Sox9 knockdowns and controls were stained with biotinylated Wisteria floribunda agglutinin (WFA) that binds N-acetylgalactosamine side chains in proteoglycans including CSPGs [18, 30]. WFA staining revealed a reduction in PNN proteoglycans in the delayed Sox9 knockdown mice caudal to the lesion compared to control mice 14 weeks post-SCI, with the
Figure 5. Reduced CSPG expression levels in Sox9 knockdown mice 14 weeks post-SCI. (A,B) Representative photomicrographs of CS56 immunostaining of longitudinal spinal cord sections centered at the T9 spinal lesion. (A) CSPG expression in control mice shown at low (left panel) and high magnification (right panel). (B) CSPG expression in Sox9 knockdown mice shown at low (left panel) and high magnification (right panel). (C) Quantification of area of CS56 immunoreactivity (area per area) in Sox9 conditional knockdown and control spinal cords. * indicates statistically significantly different from controls (p<0.05, Student’s t-test; n=6 for Sox9 knockdown mice and n=5 for controls). Scale Bars = 100 µm.
Figure 5. Reduced CSPG expression levels in Sox9 knockdown mice 14 weeks post-SCI.
Figure 6. Reduced GFAP expression levels in Sox9 knockdown mice 14 weeks post-SCI. (A-D) Representative photomicrographs of anti-GFAP immunostaining of longitudinal spinal cord sections centered at the T9 spinal lesion. (A) Control mice have abundant GFAP immunoreactivity at the lesion. (B) High magnification photomicrograph of boxed area in panel A. (C) Sox9 knockdown mice have low levels of GFAP immunoreactivity at the lesion. (D) High magnification photomicrograph of boxed area in panel C. (E) Quantification of area of GFAP immunoreactivity (area per area) in Sox9 conditional knockdown and control spinal cords. * indicates statistically significantly different from controls (p<0.05, Student's t-test; n=6 for Sox9 knockdown mice and n=5 for controls). Scale Bars = 100 µm.
Figure 6. Reduced GFAP expression levels in Sox9 knockdown mice 14 weeks post-SCI.
most notable reduction occurring in the layer VIII interneuron pool of the lumbar enlargement (Fig. 7).

**Delayed Sox9 knockdown mice display increased 5-HT immunoreactivity caudal to the lesion site following SCI**

Serotonergic projections from the Raphe Nuclei synapse in the ventral horn to modulate motor activity [31], and the loss of these serotonergic inputs to the ventral horn leads to decreased motor function [31]. To evaluate whether the improvements in hind limb function seen in the delayed Sox9 knockdown mice could be attributed to increased ventral horn serotonergic input in comparison to control mice, we performed 5-HT immunostaining on spinal cord cross-sections sections taken from 0.8 mm - 1.6 mm caudal to the lesion epicenter in the delayed Sox9 knockdowns and controls 14 weeks after SCI. The delayed Sox9 knockdown mice demonstrated a statistically significant increase in 5-HT immunoreactivity in the ventral horn, compared to control mice (Fig. 8).
Figure 7. Reduced WFA staining in Sox9 knockdown mice 14 weeks post-SCI. WFA staining (red) is reduced in the lumbar enlargement ventral horn of Sox9 knockdown mice (B) compared to controls (A) 14 weeks after SCI. Sections have been counterstained a fluorescent Nissl stain, N-21479 (green). C) Quantification of area of WFA staining (area per area of interest) in Sox9 conditional knockdown and control spinal cords. * indicates statistically significant difference from controls (p<0.05, Student's t-test; n=6 for Sox9 knockdown mice and n=5 for controls). Scale Bars = 100 µm.
Figure 7. Reduced WFA staining in Sox9 knockdown mice 14 weeks post-SCI.
Figure 8. Increased 5-HT immunoreactivity caudal to the lesion in Sox9 knockdown mice 14 weeks post-SCI. Immunohistochemistry was used to detect serotonin in the spinal cord 14 weeks post-SCI. (A, B) Representative photomicrographs of sections stained for 5-HT immunoreactivity 1.2 mm caudal to the lesion epicenter from Sox9 conditional knockdown and control mice. (A) Almost no 5-HT immunoreactivity was observed caudal to the lesion in control mice. (B) 5-HT immunoreactivity was readily observed caudal to the lesion in the ventral horn and intermediolateral cell column of Sox9 knockdown mice. Insets are high power magnifications of the boxed areas in A and B. (C) Quantification of area of 5-HT immunoreactivity (area per area) in Sox9 conditional knockdown and control spinal cords. * indicates statistically significantly different from controls (p<0.05, Student's t-test; n=3). Scale Bars = 100 µm.
Figure 8. Increased 5-HT immunoreactivity caudal to the lesion in Sox9 knockdown mice 14 weeks post-SCI.
3.4 Discussion

CSPGs are present in the adult CNS [32], and following injury their expression levels increase greatly [5, 33]. In the uninjured adult CNS, CSPGs are key components of PNNs [18] whereas, in the injured CNS, CSPGs are also key components of the glial scar [14, 34]. A demonstration that PNN CSPGs limit plasticity comes from studies in the development of ocular dominance columns. Depriving rats of visual input from one eye by lid suturing until adulthood skews ocular dominance toward the non-deprived eye. Reverse lid suturing when coupled with chondroitinase treatment of the visual cortices of these rats allows normalization of ocular dominance columns and visual function [35]. The inhibitory effect of glial scar CSPGs on recovery has been provided by numerous studies that demonstrate that chondroitinase treatment at the scar increases structural plasticity in the lesion and improves neurological outcomes in rodent models of SCI [8, 9, 13, 36]. The inhibitory effect of PNN CSPGs distant to the lesion has been demonstrated by the application of chondroitinase at the level of the cuneate nucleus in the brain stem following an ipsilateral dorsal column transection at C6-C7. This chondroitinase treatment resulted in reduced PNN CSPGs and enhanced collateral sprouting of spared afferents [37]. Thus, CSPGs in the uninjured and injured nervous system limit plasticity.

Using a tamoxifen-inducible line of Sox9 knockdown mice we previously demonstrated that Sox9 ablation prior to SCI leads to significant reduction in CSPG levels at the lesion epicenter and in PNN matrix distant to the lesion [22]. These changes in CSPG levels correlated with improved locomotor recovery in the delayed Sox9 knockdown mice. In this previous study we administered tamoxifen daily by gavage for one week and then allowed one week for tamoxifen to clear the system. This experimental protocol avoided any confounding effects of tamoxifen on recovery from SCI, and maximized the possible effects of reduced SOX9
expression by employing a pre-injury ablation strategy. However the disadvantage of this protocol was that it did not test the potential clinical value of an anti-SOX9 strategy as that requires an experimental protocol that ablates or inhibits Sox9 expression after SCI.

In the present study we administered tamoxifen daily for 1 week beginning at 7 days after SCI. Our time course evaluation of Sox9 mRNA levels in the injured spinal cords of tamoxifen-treated control and knockdown mice indicates that significant decreases in Sox9 mRNA levels were not achieved until 6 days after the first tamoxifen administration. This probably reflects the time taken for Cre activation by tamoxifen, and the half-life of Sox9 mRNA. We note that Sox9 mRNA levels continued to decline relative to controls from 13 days after SCI to 6 weeks after SCI indicating that maximal Sox9 ablation was reached sometime after 13 days post-injury. As SOX9 protein levels would not be expected to decline before Sox9 mRNA levels, SOX9 activity probably began to decline at approximately 2 weeks post-SCI. This is a time after astrocyte activation and scar deposition has begun, and thus is a rigorous test of whether Sox9 ablation can reduce CSPG levels in the glial scar after its formation has commenced. Since this subacute time period after SCI is a window of time at which SCI patients might reasonably be expected to undergo therapeutic interventions, this experimental protocol enabled us to evaluate whether potential SOX9 inhibitors could yield beneficial effects when applied within a clinically achievable time frame.

To evaluate the effect of Sox9 ablation after SCI on SOX9 target gene expression, we measured the mRNA levels of Sox9, gfap, neurocan and aggregan in a 5 mm section of spinal cord centered on the lesion at 6 weeks post-SCI. As expected Sox9 mRNA levels were approximately 75% lower in the lesions of delayed knockdown mice compared to controls. The reduction in Sox9 mRNA levels was accompanied by a similar reduction in SOX9 protein in
these lesions. The reduction in SOX9 levels were also accompanied by reduction in the mRNA and protein levels of GFAP, neurocan and brevican. Thus despite the delayed time course of Sox9 ablation, by 6 weeks post-SCI the delayed knockdown lesions had significantly lower CSPG and GFAP levels compared to controls suggesting that these lesions had less astrocyte activation and less scar deposition.

To evaluate the effect of Sox9 ablation after SCI on locomotor recovery we performed open field locomotor testing weekly and quantified hind limb function using the BMS [29]. In addition, as a more general indication of mobility, we also measured the total distance traveled by each mouse in a 2 h period using rodent activity boxes at 1, 6 and 14 weeks after SCI. The open field testing demonstrated that whereas the locomotor recovery in spinal cord-injured Sox9$^{\text{flox/flox}}$ and Sox9$^{\text{flox/flox};\text{Cre}}$ mice were indistinguishable for the first two weeks of recovery (i.e. before tamoxifen administration) Sox9$^{\text{flox/flox};\text{Cre}}$ mice began to show improved hind limb function thereafter. The rodent activity box data supported these findings and indicated that at 14 weeks post-SCI the delayed Sox9 knockdown mice traverse about twice the distance of control mice and are not different from uninjured Sox9 ablated or wild type mice. The degree of improvement in locomotor function as assessed by the BMS scores may seem modest and stands in contrast to the more obvious improvement in mobility as measured using the rodent activity boxes. This indicates that whereas the spinal cord-injured Sox9 knockdown mice are much more mobile than the spinal cord-injured controls their method for ambulation is abnormal and does not fully translate into greater BMS scores.

We have previously suggested that improved locomotor recovery in conditional Sox9 knockdown mice could potentially be explained by one or more of the following: a muted inflammatory response, altered neural stem cell behavior or increased structural neuroplasticity.
[22]. We suggested that the muted inflammatory response could be due to reduced astrocyte activation in the Sox9 knockdown mice as evidenced by their reduced GFAP expression. However as the most damaging inflammatory response in SCI is found to occur within the first hours and days of SCI [38] it would seem unlikely that a muted inflammatory response commencing at about 2 weeks after SCI could likely account for the improved recovery observed in the delayed Sox9 knockdowns described in the present study. The possibility that improved locomotor recovery observed in Sox9 knockdown mice is due to altered neural stem cell behavior rests on studies demonstrating that SOX9 directs stem cell fate down a glial lineage [39, 40]. Thus, Sox9 ablation could result in the generation of fewer astrocytes and more neurons after SCI and lead to better outcomes. We are currently testing this hypothesis using a tamoxifen-inducible neural stem cell-specific line of Sox9 knockdown mice.

The most likely explanation for improved locomotor recovery in Sox9 conditional knockdown mice is that the reductions in SOX9 activity resulted in lower CSPG levels in the glial scar and in PNNs which permitted increased structural neuroplasticity. The importance of developing methods to reduce CSPG expression both at and far from the lesion site has been suggested by others based on increased CSPG expression at these sites after SCI [41]. We have shown that Sox9 ablation by tamoxifen administration starting at 1 week after SCI results in reduced Sox9 mRNA levels as early as 13 days after SCI and reduced levels of Sox9, GFAP, neurocan and aggrecan mRNA and protein levels at the lesion by 6 weeks after SCI. Immunohistochemistry demonstrated reduced levels of GFAP and CSPGs at the lesion site in delayed Sox9 knockdowns at 14 weeks post-SCI. Reductions in PNN matrix in delayed Sox9 knockdown mice was demonstrated in the ventral horn of the lumbar enlargement by staining with WFA, a lectin that binds N-acetylgalactosamine side chains in proteoglycans including
CSPGs [18, 30]. We suggest that the reductions in CSPG levels in the Sox9 conditional knockdown mice removes the limits on neuroplasticity that these matrix molecules normally impose and accounts for the improved outcomes in the Sox9 conditional knockdown mice. We further suggest that the reduction in PNN CSPGs, more than the reduction in glial scar CSPGs, underlies the improved outcomes as long-range axonal growth from above to below the lesion is less likely to result in functional, productive circuitry than short range reactive sprouting of spared fibers. Evidence for increased neuroplasticity in delayed Sox9 knockdown mice comes from the demonstration of increased 5-HT immunoreactivity at the lumbar enlargement in the ventral horns of these animals.

We have previously shown that Sox9 ablation before SCI results in reduced CSPG levels in the spinal cord and improvements in motor function post-SCI [22]. The present study extends our previous work by demonstrating that Sox9 ablation in the subacute period after SCI also reduces CSPG levels in the injured spinal cord and yields improvements in locomotor function. These findings are supported by previous work demonstrating that chondroitinase treatment 2 weeks after SCI improves recovery in spinal cord-injured mice [42]. Thus, as a general approach, increasing neuroplasticity may be a therapeutic option well after SCI. These results suggest that Sox9 inhibition is a clinically viable and practical therapeutic strategy for the treatment of SCI.
3.5 Acknowledgments

The Sox9<sup>flax/flax</sup> mice were kindly provided by Dr. Andreas Schedl. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the International Foundation of Research in Paraplegia (IFP). WMM was supported by a doctoral scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC).

3.6 Author Disclosure Statement

A.B. holds a patent on SOX9 inhibition as a target for regeneration in the nervous system. No competing financial interests exist for W.M.M., or E.M.Y.

3.7 Supplementary Table 1. Two Way ANOVA Summary Table

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3.8 References


Chapter 4: Sox9 knockdown promotes neuroplasticity after spinal cord injury

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Running title: Sox9 knockdown promotes neuroplasticity after spinal cord injury

Keywords: SOX9, spinal cord injury, neuroplasticity, CSPG, regeneration, perineuronal nets
The absence of axonal regeneration after spinal cord injury (SCI) has been attributed to the up-regulation of axon-repelling molecules present in the glial scar that forms post-SCI. Amongst the most important of the inhibitory molecules in the scar are chondroitin sulfate proteoglycans (CSPGs) produced by reactive astrocytes that respond to the injury. We have previously identified the transcription factor SOX9 as a key regulator of CSPG production both \textit{in vitro} and \textit{in vivo}. \textit{Sox9} conditional knockdown (KO) mice display decreased CSPG expression and improved hind limb function post-SCI. Herein we investigated sparing, long-range regeneration and reactive sprouting as possible explanations for the improved locomotor outcomes in \textit{Sox9} KO mice after SCI. Retrograde tract-tracing studies failed to reveal any evidence of increased sparing or of long-range regeneration in the \textit{Sox9} KO mice compared to controls. However caudal to the lesion site we found evidence of increased neuroplasticity as indicated by increased levels of the presynaptic markers synaptophysin and vesicular glutamate 1 transporter (VGLUT1) and by increased serotonin immunoreactivity. These findings were supported by anterograde tract-tracing experiments that demonstrated increased reactive sprouting caudal to the lesion after SCI. The increased neuroplasticity after SCI in \textit{Sox9} KO mice highlights the clinical potential of SOX9 antagonists as a treatment strategy for SCI.
4.1 Introduction

Spinal cord injury (SCI) is a catastrophic event that often results in the loss of mobility and sensation below the injury site, as well as impaired organ function and sensitivity to pain. The up-regulation of axon-repelling molecules in the glial scar that forms post-SCI leads to an absence of axonal regeneration. One of the key inhibitory factors preventing regeneration in this glial scar is the chondroitin sulfate proteoglycan (CSPG) family of extracellular matrix molecules [1-3]. CSPGs inhibit axonal regeneration by imposing both a physical and molecular barrier preventing axonal passage. CSPGs sterically inhibit access to substrate adhesion molecules [4], and receptor protein tyrosine phosphatase sigma (RPTPσ) present on axonal growth cones causes growth cone collapse on interaction with CSPGs [5-7]. CSPGs also play a critical role in the development of the central nervous system (CNS) as key components of perineuronal nets (PNNs). PNNs are highly condensed extracellular matrix structures which surround the cell bodies and dendrites of some classes of neurons [8]. The function of CSPGs in PNNs is to stabilize synapses during development by preventing axonal sprouting onto inappropriate targets after appropriate connections have been made, and thus modulate neuroplasticity [1, 9-12]. Following SCI, expression levels of CSPGs dramatically increase both in the glial scar at the site of injury and in distant PNNs [3, 13, 14].

Specific CSPGs have been shown to inhibit neurite outgrowth including: NG2 [15], versican [16], neurocan [17], brevican [18] and phosphocan [19]. All of these CSPGs rely on the same enzymes, xylosyltransferase-I and -II (XT-I, XT-II) and chondroitin 4-sulfotransferase (C4ST), to add the axon-repelling chondroitin sulfate side chains to their core proteins [2, 20, 21]. These chondroitin sulfate side chains play a crucial role in axon repulsion as their digestion by treatment with the enzyme chondroitinase [22], or interference with their synthesis by
inhibiting XT-I [23], increases axonal regeneration in rodent models of SCI. Thus, CSPGs are a potential target for therapeutics focused on improving recovery post SCI.

We have previously identified SOX9 as a transcription factor that up-regulates the expression of CSPG synthesizing enzymes XT-I, XT-II and C4ST in reactive astrocytes [24], and hypothesized that SOX9 inhibition would lead to decreased CSPG expression, a lesion microenvironment more permissive to neuroregeneration, and improved neurological recovery after CNS injury. We have demonstrated that following SCI, Sox9 KO mice exhibit reduced expression of known SOX9 target genes including: XT-I, Collagen 2a, GFAP (glial fibrillary acidic protein, a marker of astrocyte activation) and three CSPG core proteins (aggrecan, brevican and neurocan) [24, 25]. This reduction in mRNA expression was accompanied by reductions in CSPG protein levels both in the glial scar and in peri-neuronal nets distant to the injury [25]. In addition to reduced CSPG levels, Sox9 KO mice also displayed improved hindlimb functional recovery as assessed by the Basso Motor Scale (BMS) and overall locomotor activity as assessed by activity boxes [25]. Finally, the Sox9 KO mice displayed increased serotonin immunostaining caudal to the injury site in the intermediolateral cell column as well as the ventral horn.

In the present study we investigated sparing, long-range regeneration and reactive sprouting as possible explanations for the improved locomotor outcomes in Sox9 KO mice after SCI. We herein report that retrograde tract-tracing studies failed to reveal any evidence of increased sparing or of long-range regeneration in the corticospinal, rubrospinal, reticulospinal, vestibulospinal tracts or long descending propriospinal projections in the Sox9 KO mice. However, Sox9 KO mice displayed increased synaptic plasticity caudal to the lesion as Sox9 KO mice displayed increased levels of the presynaptic markers synaptophysin and vesicular
glutamate 1 transporter (VGLUT1), as well as increased serotonin immunoreactivity. Anterograde tract tracing studies support the immunohistochemical evidence for increased reactive sprouting below the level of the lesion. The data presented herein describes the mechanism by which conditional Sox9 KO mice display improved hind limb function and locomotor activity post SCI, and suggest the potential utility of an anti-SOX9 treatment for SCI.

4.2 Materials and Methods

Sox9 conditional knockdown (KO) mice

Mice homozygous for floxed Sox9 (exons 2 and 3 of Sox9 surrounded by loxP sites) alleles [26] and heterozygous for Cre recombinase fused to the mutated ligand binding domain of the human estrogen receptor (ER) under the control of a chimeric cytomegalovirus immediate-early enhancer/chicken β-actin promoter [27] (Sox9\(^{\text{floxflox}}\);\(\text{CAGGCreER}\) referred to as Sox9\(^{\text{floxflox}}\);\(\text{Cre}\)) were used as Sox9 KO animals. The mutated ER ligand binding domain of the fusion protein binds tamoxifen [28] allowing for Cre transport into the nucleus where it excises \(\text{loxP}\)-flanked Sox9 DNA [27]. Tamoxifen-treated Sox9\(^{\text{floxflox}}\) littermates (not carrying the Cre transgene) served as control animals (expressing normal levels of Sox9). Tamoxifen (Sigma Aldrich, St. Louis, Missouri) was administered at 3 mg/20 g mouse by oral gavage to all Sox9\(^{\text{floxflox}}\);\(\text{Cre}\) and Sox9\(^{\text{floxflox}}\) littermates starting 14 days prior to SCI, once per day, for 7 days.

Animals were genotyped by polymerase chain reaction (PCR) analysis using the following primers:

Sox9\(^{\text{floxflox}}\) allele: 5’-ACACAGCATAGGCTACCTG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

Sox9\(^{\text{wildtype}}\) allele: 5’-GGGGTTGTCTCCTTCAGAG-3’ and
5’- TGGTAATGAGTCATACACAGTAC-3’.

Sox9\textit{knockdown} allele: 5’-GTCAAGCGACCACATG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

\(\text{Cre}^+\) allele: 5’-CAATTTACTGACCCTACAC-3’ and 5’-AGCTGGCCCAAATGTGCTG-3’.

**Spinal cord injury**

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the Guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. \(\text{Sox}^9\text{fl}ax^{/}/\text{Cre}\) and \(\text{Sox}^9\text{fl}ox^{/}/\text{fl}ox\) mice were anesthetized with 100 mg/kg ketamine: 5 mg/kg xylazine. The spinal cord was stabilized at vertebra T7 and T9 with forceps, and the 9th thoracic spinal cord segment (T9) was exposed by a T8 dorsal laminectomy. The T9 spinal segment was injured using the Infinite Horizon Impactor to deliver a 70 kdyne contusion injury with a 1 s dwell time (displacement range: 500-900 \(\mu\)M) (Precision Systems and Instrumentation, Fairfax, Virginia). Following SCI the mice were housed individually. Baytril (25 mg/kg, Bayer, Toronto, Ontario, Canada) and buprenorphine (0.01 mg/kg, Schering-Plough, Hertfordshire, UK) were injected subcutaneously twice daily for 3 days post-SCI. Bladders were emptied manually twice daily for the duration of the experiment. The experimental timeline is shown in Figure 1.
Figure 1. Experimental Timeline. Sox9<sup>flox/flox</sup>;Cre and Sox9<sup>flox/flox</sup> control mice received daily administrations of tamoxifen for one week. Following a week for cre mediated recombination and tamoxifen clearance the mice were subjected to a SCI at T9 using the infinite Horizons Impactor. 1 week post-SCI a subset of mice underwent a second T10 spinal cord transection along with the insertion of fluorogold soaked gel foam. These mice were sacrificed at week 3 to assess axonal sparing post-SCI. 8 weeks post-SCI a subset of mice underwent a second T10 spinal cord transection along with the insertion of fluorogold soaked gel foam. These 8 week fluorogold mice were sacrificed at week 10 to assess long range regeneration post-SCI. 8 weeks post-SCI a subset of mice underwent BDA injections into the primary motor cortex. These 8 week BDA mice were sacrificed at week 10 to assess reactive sprouting caudal to the lesion. The BDA mice were also used for WFA, synaptophysin, VGLUT1, VGAT, and serotonin immunohistochemical analyses.
Figure 1. Experimental Timeline.
Spinal cord sectioning

Groups of mice were sacrificed over the 10 week post-SCI timeline by deep anesthesia with 50 mg/kg ketamine: 5 mg/kg xylazine, and cardiac perfusion with 20 ml of saline at pH 7.4 followed by 20 ml 4% paraformaldehyde (4% PFA in 0.1 M phosphate buffer at pH 7.4). Spinal cords were dissected and post-fixed for 2 h in 4% PFA followed by cryoprotection in 20% sucrose in 0.1 M phosphate buffer at pH 7.4 at 4°C overnight. Spinal cords were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A. Inc, Torrance, California), frozen over dry ice, and stored at -80°C overnight. Frozen cords were then sectioned at 16 µm using a cryostat, and serially thaw-mounted on Superfrost™ glass slides (Fisher Scientific Company, Ottawa, Canada).

Immunohistochemistry

Immunohistochemistry was conducted at the end of the study, 10 weeks post SCI, using the primary antibodies listed in Table 1. Slides were rinsed in PBS and treated with 5% normal goat serum and 0.1% triton-X-100 in phosphate buffered saline (PBS) at room temperature for 1 h and then incubated with the primary antibodies in a humidified chamber at 4°C overnight. Sections to be stained for PNNs were washed in PBS 3 x 10 min, and incubated with biotinylated Wisteria Floribunda Agglutinin (WFA at 1:1000, Sigma Aldrich) for 1 h at room temperature. Biotinylated WFA was detected by streptavidin conjugated Alexa-Fluor 594 (1:500 Invitrogen, Carlsbad, California) for 45 min at room temperature. Sections were stained for serotonin with an antibody against 5-hydroxytryptamine (5-HT) (1:500 ImmunoStar, Hudson, Wisconsin). Anti-5-HT was detected by Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:500, Invitrogen) for 1 h at room temperature. Sections were stained for synaptophysin (1:150 Sigma Aldrich). Anti-
synaptophysin was detected by Alexa-Fluor 488-conjugated goat anti-mouse IgG (1:500, Invitrogen) for 1 h at room temperature. Sections were stained for VGLUT1 (1:200 Synaptic Systems, Goettingen, Germany). Anti-VGLUT1 was detected by Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:500, Invitrogen) for 1 h at room temperature. Sections were stained for VGAT (1:200 Synaptic Systems). Anti-VGAT was detected by Alexa-Fluor 488-conjugated goat anti-mouse IgG (1:500, Invitrogen) for 1 h at room temperature. All fluorescently labeled slides were washed in PBS and coverslips applied with ProLong Gold Anti-Fade mounting medium (Invitrogen).

Table 1. List of primary antibodies and stains used for spinal cord staining

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<tr>
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<tr>
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<td>Rabbit IgG</td>
<td>ImmunoStar, Hudson, Wisconsin</td>
</tr>
</tbody>
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Retrograde labeling study

Either 1 week or 8 weeks post-SCI mice were anaesthetized and underwent a second dorsal laminectomy one segment caudal to the original dorsal contusion, a T9 dorsal laminectomy exposing the 10th thoracic spinal cord segment (T10). The T10 spinal cord was fully transected by scalpel and a gel foam pledget soaked in 4% hydroxystilbamidine (a
fluorescent molecule responsible for retrograde transport in the common retrograde tracer Fluoro-Gold [29]) (4% w/v in saline; Invitrogen) was inserted into the T10 site. The incisions were sutured and the mice returned to animal housing. Two weeks later the mice underwent cardiac perfusion with 4% paraformaldehyde after which their spinal cords were cryosectioned in cross section (16 μm thick sections) at the cervical enlargement, and their brains were cryosectioned in coronal section (30 μm thick sections). Sections from the cervical enlargement as well as several areas of the brain (primary motor cortex, red nucleus, reticular formation, and vestibular formation), were directly visualized for retrograde tracer labeling.

**Anterograde labeling study**

Eight weeks post-injury mice were re-anesthetized and their heads stabilized in a stereotaxic frame. A burr hole (1.5 mm in diameter) was made in the skull overlying the left sensorimotor cortex. BDA (biotinylated dextran amine, 10,000 d, Molecular Probes, Invitrogen) was injected by Hamilton syringe with a 33G needle (0.4 μl of 10% BDA suspended in PBS) at a depth of 0.5 mm from the cortical surface in the hindlimb area of the motor cortex at 4 sites centered on +1.5mm lateral, -1mm posterior to bregma (+1mm lateral, -0.5mm posterior, +2mm lateral, -0.5mm posterior, +1mm lateral, -1.5mm posterior; +2mm lateral, -1.5mm posterior) [30] to label corticospinal neurons. The syringe remained in position for 1 min after BDA injection. Two weeks after BDA injections, mice underwent cardiac perfusion with 4% paraformaldehyde and their spinal cords were cut in cross section (16 μm thick sections) at C4, and in the lumbar enlargement (L1). Sections for BDA fiber staining in the cervical enlargement were incubated for 45 min with avidin-peroxidase conjugate (Elite Kit, Vector laboratories, Burlingame, California) at room temperature, and the signal visualized by peroxidase diaminobenzine (DAB,
Invitrogen). All DAB staining was conducted with a 2-min DAB reagent incubation time and processed at the same time. The number of BDA-labeled fibers at C4 were counted in both uninjured and SCI mice, and used to assess sprouting rostral to injury as well as BDA labeling efficiency. Sections for the quantification of BDA puncta surrounding motor neurons in the ventral horn cervical enlargement or lumbar enlargement by high magnification confocal imaging were stained for 45 min with streptavidin conjugated Alexa-Fluor 594 (1:500 Invitrogen, Carlsbad, California) at room temperature, and co-stained with anti-VGLUT1 and anti-NeuN. BDA and VGLUT1 labeled puncta surrounding motor neurons in the ventral horn of the cervical and lumbar enlargements were compared between Sox9 KO and control mice to assess reactive sprouting both rostral and caudal to the injury site.

**Quantification of retrograde tracer, anterograde tracer, WFA, Serotonin, Synaptophysin, VGLUT1, and VGAT immunostaining**

Retrograde tracer labeled neurons were counted individually in the primary motor cortex, red nucleus, reticular formation, and vestibular formation using 30 μm thick cross-sections spaced 300 μm apart spanning the relevant structures (~1.8mm bregma - ~0.3mm bregma, ~3mm bregma - ~3.9mm bregma, ~5.1mm bregma - ~7.2mm bregma and ~6mm bregma - ~6.9mm bregma for the primary motor cortex, red nucleus, reticular formation, and vestibular formation respectively). Long range pro-priospinal neuron retrograde tracer labeling was evaluated in the cervical enlargement (C4) using ten 16 μm thick spinal cord cross-sections spaced 160 μm apart from each area.

The number of BDA labeled fibers were counted across ten 16 μm thick spinal cord cross-sections each spaced 160 μm apart from the cervical enlargement (C4). BDA labeling was
assessed throughout the grey matter, and fibers measuring at least 5 μm in length were counted as real BDA labeled fiber staining. BDA labeling was robust at C4 in both uninjured and injured mice, with no statistical difference between Sox9 KO and control mouse labeling. BDA puncta surrounding motor neurons was evaluated in both the cervical enlargement and the lumbar enlargement by imaging five high magnification z-stacks (10 slices 0.2 μm apart) per mouse taken at NeuN stained motor neurons defined by their position in Rexed laminae layer 8 or 9, as well as their size and appearance, at 63x with a 3x zoom by a Zeiss LSM-510-Meta confocal microscope. Positive staining was identified as individual puncta measuring at least 1.5 μm in diameter as quantified by the spots algorithm in the Imaris x64 7.0 software package (Bitplane USA, South Windsor, CT).

The area of WFA and 5-HT immunoreactivity was examined just caudal to the lesion site (T10) as well as in the lumbar enlargement (L1) using ten 16 μm thick spinal cord cross-sections spaced 160 μm apart from each area. A single pre-set area of interest was used to define all ventral horn regions in all cords across sections from both Sox9 KO and control animals. Positive WFA and 5-HT staining was quantified using ImagePro Plus Software (Media Cybernetics Inc, Bethesda, Maryland) using a threshold which identified positive signal (staining above background levels).

Synaptophysin, VGLUT1, and VGAT were examined at the lumbar enlargement (L1) using ten 16 μm thick cross-sections 160 μm apart captured by Zeiss LSM-510 confocal microscope. A single pre-set area of interest was used to define all ventral horn regions in all cords across sections from both Sox9 KO and control animals. Positive staining was quantified using ImagePro Plus Software (Media Cybernetics Inc, Bethesda, Maryland) using a threshold which identified positive signal (staining above background levels). Five high magnification
ventral horn motor neuron z-stack images (10 slices 0.2 μm apart) per mouse were taken at 63x with a 3x zoom by a Zeiss LSM-510-Meta confocal microscope. Positive staining was identified as individual puncta measuring at least 1.5 μm in diameter as quantified by the spots algorithm in the Imaris x64 7.0 software package (Bitplane USA, South Windsor, CT). All microscopy was completed using a Olympus BX-50 epifluorescence microscope except when a Zeiss LSM-510-Meta confocal was used as indicated.

**Statistical analysis**

WFA, Synaptophysin, VGLUT1, VGAT, and 5-HT areas of immunoreactivity, as well as retrograde tracer neuronal labeling, were compared between Sox9 KO and controls using a one way ANOVA with a Neuman Keuls post hoc test. Synaptophysin, VGLUT1, VGAT, and BDA positive individual puncta counts were compared between Sox9 KO and controls using Student’s T-test. Analyses were conducted with GraphPad Prism software (GraphPad Software Inc, La Jolla, California). Statistical significance was accepted at p<0.05. Mean values are expressed ± SE.

**4.3 Results**

**Sox9 KO mice demonstrate decreased PNN matrix post-SCI**

CSPGs are a major component of the PNN that stabilizes synapses during development and limits plasticity in the adult central nervous system [1]. As Sox9 KO mice demonstrate reduced CSPG levels throughout the injury site [25], we evaluated whether they may also have reductions in their PNNs directly caudal to the injury, as well as at the distant lumbar
enlargement where descending axons synapse on the motor neurons that innervate the hind limb musculature. Cross-sections from T10 and the lumbar enlargement of both uninjured and SCI Sox9 KO and control mice were stained with biotinylated Wisteria floribunda agglutinin (WFA). WFA binds N-acetylgalactosamine side chains in proteoglycans including CSPGs [8, 31]. WFA staining revealed that spinal cord-injured Sox9 KO mice had significantly lower levels of PNN matrix in their ventral horns at T10 (a 56% reduction) and in the distant lumbar enlargement (a 58% reduction) compared to the PNN matrix in control mice 10 weeks post SCI (p<0.05 by one-way ANOVA, Figure 2). WFA staining of PNN matrix was not different between uninjured control, uninjured Sox9 KO mice or spinal cord-injured Sox9 KO mice.

**Sox9 KO mice display no evidence of altered axonal sparing or of long range axonal regeneration post-SCI**

Retrograde labeling was carried out to examine axonal sparing and long range regeneration in the Sox9 KO mice. At 1 week or 8 weeks after T9 SCI control and Sox9 KO mice underwent retrograde labeling by placing a hydroxystilbamidine-soaked pledget caudal to the lesion (at T10). Since axons can not regenerate a full segment (~3 mm) caudal to the lesion within a week of injury, the neurons labeled at 1 week after injury must have axons that were spared from injury [32, 33]. We examined retrograde labeling in the cervical enlargement for long range propriospinal neurons, as well as in the motor cortex for corticospinal tract neurons, the red nucleus for rubrospinal tract neurons, the reticular formation for reticular spinal tract neurons, and the vestibular formation for vestibulospinal tract neurons. At 1 week post SCI both Sox9 KO and control mice displayed similar significant reductions in retrograde tracer-labeled corticospinal, and reticulospinal neurons when compared to uninjured controls (Figure 3). To
Figure 2. WFA staining is reduced both just caudal to the injury site, as well as in the lumbar enlargement in Sox9 KO mice 10 weeks post-SCI. A) Perineuronal net staining in uninjured control mice at T10. B) Perineuronal net staining in uninjured Sox9 KO mice at T10. C) Perineuronal net staining in control mice just caudal to the injury site (T10). D) Perineuronal net staining in Sox9 KO mice just caudal to the injury site (T10). E) Sox9 KO mice display reduced WFA staining just caudal to the injury site (p<0.05, one way ANOVA). F) Perineuronal net staining at the ventral horn of the lumbar enlargement (L1) in uninjured control mice. G) Perineuronal net staining in the ventral horn of the lumbar enlargement (L1) in uninjured Sox9 KO mice. H) Perineuronal net staining in the ventral horn of the lumbar enlargement (L1) in control mice post-SCI. I) Perineuronal net staining in Sox9 KO mice in the ventral horn of the lumbar enlargement (L1) post-SCI. J) Sox9 KO mice display reduced WFA staining in the ventral horn of the lumbar enlargement post-SCI (p<0.05, one way ANOVA). This suggests the potential for increased plasticity in Sox9 KO mice. Scale bars = 100μm.
Figure 2. WFA staining is reduced both just caudal to the injury site, as well as in the lumbar enlargement in Sox9 KO mice 10 weeks post-SCI.
evaluate whether long-range regeneration of corticospinal, and reticulospinal axons through the lesion site might occur in the Sox9 KO mice we investigated retrograde labeling after allowing sufficient time for axonal regrowth (8 weeks after SCI). This analysis failed to demonstrate any increase in retrogradely labeled neurons (Figure 3). Similar results were obtained both 1 week and 8 weeks after SCI when evaluating retrogradely-labeled rubrospinal, vestibulospinal or propriospinal interneurons (Supplementary Figure 1).

**Sox9 KO mice display increased levels of presynaptic protein markers caudal to the lesion site post-SCI**

We predicted that increased neuroplasticity in spinal cord injured Sox9 KO mice would be most evident around lumbar motor neurons as they demonstrate reduced levels of PNN CSPGs and are deafferented by the spinal lesion. Thus we evaluated the area of immunoreactivity of presynaptic markers in the ventral horn of the lumbar enlargement in control and Sox9 KO mice. Synaptophysin has been used as a marker of synaptic density [34]. At 48 h after SCI both Sox9 KO and control mice demonstrated a similar large reduction in synaptophysin area of immunoreactivity caudal to the lesion when compared to uninjured mice (Figure 4). At 10 weeks post-injury the Sox9 KO mice demonstrated a significant 31% increase in the area of synaptophysin immunoreactivity compared to injured controls.

To characterize this neuroplasticity further, we evaluated the area of immunoreactivity of the presynaptic markers VGLUT1 and VGAT. At 48 h after SCI, spinal cord sections from both Sox9 KO and control mice had approximately the same amount of VGLUT1+ and VGAT+ area of immunoreactivity, but by 10 weeks post-SCI sections from Sox9 KO mice demonstrated a significant 39% increase in VGLUT1 immunoreactivity compared to controls (Figure 5). No
Figure 3. Sox9 KO mice do not display increased axonal sparing or long range axonal regeneration post-SCI. Uninjured mice display more fluorogold labeled corticospinal and reticulospinal tract neurons, than injured mice. Control and Sox9 KO mice do not display differing fluorogold labeling at 1 week after SCI indicating that Sox9 KO does not improve sparing post-SCI in these tracts. Control and Sox9 KO mice do not display differing fluorogold labeling at 8 weeks post-SCI indicating that Sox9 KO does not result in increased long range (at least 1 spinal segment) axonal regeneration after SCI in these tracts (p=<0.05, one way ANOVA. * indicates significantly different from the injured groups. a, b, c indicates significantly different from each other). Scale bars = 100μm. Supplementary Figure 1 contains fluorogold labeling data depicting that Sox9 KO mice also do not display increased sparing or long range axonal regeneration in cervical spinal cord propriospinal interneurons, the rubrospinal tract or the vestibular spinal tract post-SCI.
Figure 3. Sox9 KO mice do not display increased axonal sparing or long range axonal regeneration post-SCI.
**Figure 4. Sox9 KO mice display increased synaptophysin immunoreactivity in the ventral horn of the lumbar enlargement 10 weeks post-SCI.** A) Synaptophysin immunoreactivity in uninjured control mice. B) Synaptophysin immunoreactivity in uninjured Sox9 KO mice. C) Synaptophysin immunoreactivity in control mice 48 hrs post-SCI. D) Synaptophysin immunoreactivity in Sox9 KO mice 48 hrs post-SCI. E) Synaptophysin immunoreactivity in control mice 10 weeks post-SCI. F) Synaptophysin immunoreactivity in Sox9 KO mice 10 weeks post-SCI. G) Both control and Sox9 KO mice display increased synaptophysin immunoreactivity at 10 weeks post-SCI in comparison to 48 hr post-SCI, and Sox9 KO mice display significantly increased synaptophysin immunoreactivity 10 weeks post-SCI compared to control mice (p<0.05 by one way ANOVA with Neuman Keuls post-hoc test, a,b,c,* each significantly different from each other). Dotted line separates GM (grey matter) from WM (white matter). Scale bars indicate 100μm.
The lumbar mice displayed increased synaptic immunoactivity in the ventral horn of the lumbar enlargement 10 weeks post-SCI. Synaptophysin quantification showed a significant increase in immunoactivity in Sox9 KO mice compared to control mice.

**Synaptophysin quantification in the ventral horn of the lumbar enlargement**

- **Uninjured**: Control (A) > Sox9 KO (B)
- **48 hr. post-SCI**: Control (C) > Sox9 KO (D)
- **10 weeks post-SCI**: Control (E) < Sox9 KO (F)

**Statistical Analysis**:
- a: Significant difference between Control and Sox9 KO
- b: Significant difference between 48 hr. post-SCI and 10 weeks post-SCI
- *: Significant difference between 10 weeks post-SCI and 10 weeks post-SCI Sox9 KO
Figure 5. Sox9 KO mice display increased VGLUT1 immunoreactivity in the ventral horn of the lumbar enlargement 10 weeks post-SCI. A) VGLUT1 immunoreactivity in uninjured control mice. B) VGLUT1 immunoreactivity in uninjured Sox9 KO mice. C) VGLUT1 immunoreactivity in control mice 48 hrs post-SCI. D) VGLUT1 immunoreactivity in Sox9 KO mice 48 hrs post-SCI. E) VGLUT1 immunoreactivity in control mice 10 weeks post-SCI. F) VGLUT1 immunoreactivity in Sox9 KO mice 10 weeks post-SCI. G) Both control and Sox9 KO mice display increased VGLUT1 immunoreactivity at 10 weeks post-SCI in comparison to 48 hr post-SCI, and Sox9 KO mice display significantly increased VGLUT1 immunoreactivity 10 weeks post-SCI compared to control mice (p<0.05 by one way ANOVA with Neuman Keuls post-hoc test, a,b,c,* each significantly different from each other). Dotted line separates GM (grey matter) from WM (white matter). Scale bars indicate 100μm.
Figure 5. Sox9 KO mbar enlargement 10 weeks post-VGLUT1 SCI. Immunoreactivity in the ventral horn of the lumbar enlargement.
Figure 6. Control and Sox9 KO mice display similar VGAT immunoreactivity in the ventral horn of the lumbar enlargement 10 weeks post-SCI. A) VGAT immunoreactivity in uninjured control mice. B) VGAT immunoreactivity in uninjured Sox9 KO mice. C) VGAT immunoreactivity in control mice 48 hrs post-SCI. D) VGAT immunoreactivity in Sox9 KO mice 48 hrs post-SCI. E) VGAT immunoreactivity in control mice 10 weeks post-SCI. F) VGAT immunoreactivity in Sox9 KO mice 10 weeks post-SCI. G) Both control and Sox9 KO mice display increased VGAT immunoreactivity at 10 weeks post-SCI in comparison to 48 hr post-SCI (p<0.05 by one way ANOVA with Neuman Keuls post-hoc test, a,b,c each significantly different from each other). Dotted line separates GM (grey matter) from WM (white matter). Scale bars indicate 100μm.
Figure 6. Corn of the control and Sox9 KO showed similar VGAT immunoreactivity in the ventral horn of the lumbar enlargement.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Uninjured</th>
<th>48 hr. post-SCI</th>
<th>10 weeks post-SCI</th>
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<tbody>
<tr>
<td>Control</td>
<td>A</td>
<td>G</td>
<td>E</td>
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<tr>
<td>Sox9 KO</td>
<td>B</td>
<td>D</td>
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VGAT quantification in the ventral horn of the lumbar enlargement

- Uninjured
- Uninjured
- 48 hr. post-SCI
- 48 hr. post-SCI
- 10 weeks post-SCI
- 10 weeks post-SCI
differences were found in VGAT immunostaining between controls and Sox9 KO mice 10 weeks post SCI (Figure 6).

To investigate whether the increased areas of synaptophysin and VGLUT1 immunoreactivities might indicate increased number of presynaptic release sites, the number of synaptophysin and VGLUT1 immunoreactive puncta were counted 10 weeks after injury in the ventral horns of sections from the lumbar enlargements of Sox9 KO and control spinal cords using high-power magnification. Increased individual synaptophysin+ (a 52% increase, Figure 7) and VGLUT1+ (a 2.34 fold increase, Figure 8) puncta were noted in Sox9 KO mice in comparison to controls, whereas individual VGAT+ (Figure 9) puncta were found to be similar in both Sox9 KO and control mice 10 weeks post SCI.

**Sox9 KO mice display increased reactive sprouting caudal to the lesion site post-SCI**

The increased presynaptic marker immunoreactivity and puncta density observed in the lumbar enlargement of Sox9 KO mice could theoretically be explained by an increased level of protein expression or by an increased level of reactive sprouting. To investigate reactive sprouting in the spinal cord anterograde labeling experiments were performed. BDA was injected into the motor cortex of control and Sox9 KO mice at 8 weeks after SCI and then after allowing 2 weeks for anterograde transport the mice were sacrificed and BDA labeling at cervical and lumbar spinal levels was evaluated. Counting individual BDA labeled puncta around motor neurons in the lumbar enlargement demonstrated a greater than two-fold increase in BDA labeling in Sox9 KO mice compared to controls (a 2.28 fold increase, Figure 10). In support of the prediction that the increased sprouting of corticospinal axons might underlie the improved locomotor recovery, in comparison to controls the Sox9 KO mice displayed a greater
Figure 7. *Sox9* KO mice display increased pre-synaptic terminal synaptophysin positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI. A) Confocal micrograph of synaptophysin+ pre-synaptic boutons in control mice 10 weeks post-SCI. B) Confocal micrograph of synaptophysin+ pre-synaptic boutons with Imaris identified individual puncta overlayed in control mice 10 weeks post-SCI. C) Imaris identified individual synaptophysin+ puncta alone in control mice 10 weeks post-SCI. D) Confocal micrograph of synaptophysin+ pre-synaptic boutons in *Sox9* KO mice 10 weeks post-SCI. E) Confocal micrograph of synaptophysin+ pre-synaptic boutons with Imaris identified individual puncta overlayed in *Sox9* KO mice 10 weeks post-SCI. F) Imaris identified individual synaptophysin+ puncta alone in *Sox9* KO mice 10 weeks post-SCI. G) *Sox9* KO mice display significantly increased synaptophysin+ pre-synaptic boutons 10 weeks post-SCI compared to control mice (p<0.05 by Student’s T-test). Visualized with a 63x magnification objective, scale bar = 25 μm.
Figure 7. Sox9 KO mice display increased pre-synaptic terminal synaptophysin positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI.
Figure 8. Sox9 KO mice display increased pre-synaptic terminal VGLUT1 positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI. A) Confocal micrograph of VGLUT1+ pre-synaptic boutons in control mice 10 weeks post-SCI. B) Confocal micrograph of VGLUT1+ pre-synaptic boutons with Imaris identified individual puncta overlayed in control mice 10 weeks post-SCI. C) Imaris identified individual VGLUT1+ puncta alone in control mice 10 weeks post-SCI. D) Confocal micrograph of VGLUT1+ pre-synaptic boutons in Sox9 KO mice 10 weeks post-SCI. E) Confocal micrograph of VGLUT1+ pre-synaptic boutons with Imaris identified individual puncta overlayed in Sox9 KO mice 10 weeks post-SCI. F) Imaris identified individual VGLUT1+ puncta alone in Sox9 KO mice 10 weeks post-SCI. G) Sox9 KO mice display significantly increased VGLUT1+ pre-synaptic boutons 10 weeks post-SCI compared to control mice (p<0.05 by Student’s T-test). Visualized with a 63x magnification objective, scale bar = 25 μm.
Figure 8. Sox9 KO mice display increased pre-synaptic terminal VGLUT1 positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI.
Figure 9. Control and Sox9 KO mice display similar pre-synaptic terminal VGAT positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI. A) Confocal micrograph of VGAT+ pre-synaptic boutons in control mice 10 weeks post-SCI. B) Confocal micrograph of VGAT+ pre-synaptic boutons with Imaris identified individual VGAT+ puncta overlayed in control mice 10 weeks post-SCI. C) Imaris identified individual VGAT+ puncta alone in control mice 10 weeks post-SCI. D) Confocal micrograph of VGAT+ pre-synaptic boutons in Sox9 KO mice 10 weeks post-SCI. E) Confocal micrograph of VGAT+ pre-synaptic boutons with Imaris identified individual VGAT+ puncta overlayed in Sox9 KO mice 10 weeks post-SCI. F) Imaris identified individual VGAT+ puncta alone in Sox9 KO mice 10 weeks post-SCI. G) Sox9 KO mice display significantly increased VGAT+ pre-synaptic boutons 10 weeks post-SCI compared to control mice (p<0.05 by Student’s T-test). Visualized with a 63x magnification objective, scale bar = 25 μm.
Figure 9. Control and Sox9 KO mice display similar pre-synaptic terminal VGAT positive boutons in layer IX of ventral horn at the lumbar enlargement 10 weeks post-SCI.
Figure 10. High magnification images display increased BDA and VGLUT1 positive puncta around ventral horn motor neurons in the lumbar enlargement in Sox9 KO mice. Motor neurons in the ventral horn of the lumbar enlargement stained for BDA+ puncta, VGLUT1+ puncta, and BDA-VGLUT1 co-localized puncta viewed by confocal z-stack. BDA confocal z-stack (A) and Imaris identified individual BDA+ puncta (B) in control mice. BDA confocal z-stack (C) and Imaris identified individual BDA+ puncta (D) in Sox9 KO mice. E) Motor neurons in the ventral horn of the lumbar enlargement displayed increased BDA+ puncta in Sox9 KO mice (p=<0.05, Student’s t-test). VGLUT1 confocal z-stack (F) and Imaris identified individual VGLUT1+ puncta (G) in control mice. VGLUT1 confocal z-stack (H) and Imaris identified individual VGLUT1+ puncta (I) in Sox9 KO mice. J) Motor neurons in the ventral horn of the lumbar enlargement displayed increased VGLUT1+ puncta in Sox9 KO mice (p=<0.05, Student’s t-test). NeuN (blue) BDA-VGLUT1 confocal z-stack (K) and Imaris identified individual BDA-VGLUT1+ co-localized puncta (L) in control mice. NeuN (blue) BDA-VGLUT1 confocal z-stack (M) and Imaris identified individual BDA-VGLUT1+ co-localized puncta (N) in Sox9 KO mice. O) Motor neurons in the ventral horn of the lumbar enlargement displayed increased numbers of BDA-VGLUT1 co-localized puncta in Sox9 KO mice (p=<0.05, Student’s t-test). Scale bars indicate 10μm.
Figure 10. High magnification images display increased BDA and VGLUT1 positive puncta around ventral horn motor neurons in the lumbar enlargement in Sox9 KO mice.

<table>
<thead>
<tr>
<th></th>
<th>BDA Confocal Photomicrograph</th>
<th>BDA Imaris Identified Individual puncta</th>
<th>VGLUT1 Confocal Photomicrograph</th>
<th>VGLUT1 Imaris Identified Individual puncta</th>
<th>BDA-VGLUT1 Co-localized Imaris Identified Individual puncta</th>
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<tr>
<td>Control</td>
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<td>Sox9 KO</td>
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Average number of BDA+ puncta around ventral horn motor neurons sampled in the lumbar enlargement

Average number of VGLUT1+ puncta around ventral horn motor neurons sampled in the lumbar enlargement

Average number of BDA-VGLUT1 co-localized puncta around ventral horn motor neurons sampled in the lumbar enlargement
than three-fold increase in the number of BDA-labeled puncta that were co-localized with VGLUT1 (a 3.03 fold increase, Figure 10).

BDA labeling in the cervical enlargement of uninjured Sox9 KO and control mice was investigated to evaluate whether Sox9 ablation on its own (without an accompanying injury) might lead to increased reactive sprouting. BDA labeling of axons in uninjured mice was found to be the same in both Sox9 KO and control mice (Supplemental Figure 2). To evaluate BDA labeling distant to a denervated site we investigated BDA labeling around ventral horn motor neurons in the cervical enlargement following SCI. In comparison to control mice, Sox9 KO mice did not display altered BDA+ puncta, VGLUT1+ puncta, or BDA-VGLUT1 co-localized puncta, around ventral horn motor neurons in the cervical enlargement following SCI (Supplementary Figure 3).

**Sox9 KO mice display increased 5-HT immunoreactivity caudal to the lesion site after SCI**

Serotonergic projections from the Raphe Nuclei synapse in the ventral horn and are believed to directly modulate motor activity [35]. Injuries which lead to a reduction in these serotonergic inputs to the ventral horn result in decreased motor function [36]. To evaluate whether the improvements in hind limb function seen in Sox9 KO mice could be the result of increased ventral horn serotonergic input, we investigated serotonin immunoreactivity in spinal cord cross-sections sections taken from T10 and the lumbar enlargement of both uninjured and SCI Sox9 KO and control mice. Following injury both Sox9 KO and control mice demonstrate decreased serotonin immunoreactivity compared to uninjured controls, however, Sox9 KO mice demonstrate a statistically significant increase in serotonin immunoreactivity in the ventral horn
at spinal levels T10 (a 2.19 fold increase) and L1 (a 2.39 fold increase), compared to control mice 10 weeks post SCI (p<0.05 by one-way ANOVA, Figure 11).
Figure 11. *Sox9* KO mice display increased serotonin immunoreactivity both just caudal to the injury site and in the ventral horn of the lumbar enlargement 10 weeks post-SCI. A) Serotonin immunoreactivity in uninjured control mice at T10. B) Serotonin immunoreactivity in uninjured *Sox9* KO mice at T10. C) Serotonin immunoreactivity in control mice just caudal to the injury site (T10). D) Serotonin immunoreactivity in *Sox9* KO mice just caudal to the injury site (T10). E) Just caudal to the injury site, *Sox9* KO mice display increased serotonin immunoreactivity in comparison to control mice post-SCI (p<0.05, one way ANOVA). F) Serotonin immunoreactivity at the ventral horn of the lumbar enlargement (L1) in uninjured control mice. G) Serotonin immunoreactivity in the ventral horn of the lumbar enlargement (L1) in uninjured *Sox9* KO mice. H) Serotonin immunoreactivity in the ventral horn of the lumbar enlargement (L1) in control mice post-SCI. I) Serotonin immunoreactivity in *Sox9* KO mice in the ventral horn of the lumbar enlargement (L1) post-SCI. J) *Sox9* KO mice display increased serotonin immunoreactivity in the ventral horn of the lumbar enlargement post-SCI (p<0.05, one way ANOVA, a,b,c are significantly different from each other). Scale bars = 100μm.
Figure 11. Sox9 KO mice display increased serotonin immunoreactivity both just caudal to the injury site and in the ventral horn of the lumbar enlargement 10 weeks post-SCI.
4.4 Discussion

Recovery following SCI is significantly impeded by the CSPG-rich inhibitory extracellular matrix that serves to prevent neuroplasticity both at the lesion site as well as distant to the lesion [37-39]. Degradation of CSPG side chains by administration of the bacterial enzyme chondroitinase results in enhanced regeneration following SCI in rodents [4, 13, 22, 40], as well as improved skilled locomotor function in adult cats following T10 hemisection [41]. We previously proposed that SOX9 inhibition could serve as an alternate strategy for reducing CSPG levels in the injured spinal cord by reducing the expression of CSPG biosynthetic enzymes and core proteins [24, 25]. This proposition was supported by our previous study demonstrating that conditional Sox9 ablation reduces CSPG levels in the injured spinal cord and improves hind limb function after SCI [25]. However that study did not address the mechanism through which Sox9 ablation resulted in improved locomotor recovery.

Sox9 ablation could lead to better locomotor outcomes after SCI for a variety of reasons. First, we have previously demonstrated that Sox9 KO is associated with a concomitant reduction in GFAP expression [25]. Astrocytes activated at the time of SCI display upregulated GFAP as well as increased cytokine production and stimulate the inflammatory response [42, 43]. As Sox9 KO mice display reduced GFAP, they may have a reduced inflammatory response post-SCI compared to controls. This could result in increased sparing of descending axons at the time of injury, and perhaps the preservation of injured nervous tissue. Alternatively, reduced CSPG levels at the glial scar [25] could create an environment more permissive to long-range axonal regeneration through the lesion site. Finally reduced CSPG levels in PNNs [25] could provide an environment that promotes reactive sprouting. Herein we evaluated each of these potential mechanisms of neuroplasticity in the Sox9 KO mice.
We assessed neuronal sparing by labeling axons with retrograde tracer one segment caudal to the original injury site one week after SCI. It has been estimated that roughly 5 days are required for the proximal stump of a severed axon to prepare for growth, and axonal growth rates are suggested to be 0.25 mm/day through an injury site [32, 33]. Thus, 1 week of recovery is not enough time for regeneration through the lesion to the site of retrograde tracer labeling (a distance of ~3mm). Thus at 1 week post-SCI only axons spared from injury should be available for retrograde labeling. The absence of significant difference in the number of neurons with descending projections labeled in this way between Sox9 KO and control mice suggests that increased sparing of axons through the lesion site in Sox9 KO mice is not the explanation for their improved locomotor recovery after SCI.

To assess the possibility of long-range regeneration in spinal cord-injured mice we carried out retrograde labeling at 8 weeks post-SCI, a time point at which one would predict that sufficient time has elapsed from the injury to allow for regeneration through the lesion [32, 33]. This labeling study failed to reveal any evidence for long range regeneration in the corticospinal, reticulospinal, rubrospinal, vestibulospinal or long range propriospinal tracts within the Sox9 KO mice. This may not be all that surprising as a considerable number of anti-regenerative molecules likely still remain at the lesion site. Semaphorins, ephrins, netrins, slit, Nogo, myelin-associated glycoprotein, repulsive guidance molecule, and oligodendrocyte myelin glycoprotein are all expressed after injury in the glial scar and induce axonal growth cone collapse [3, 44-50]. Thus the failure to demonstrate long-range regeneration in the Sox9 KO mice after SCI may be due to the expression of various inhibitors to axon growth that are not affected by Sox9 ablation. With that said, perhaps the reason that long range regeneration does not contribute to natural recovery post-SCI is the difficulty a regenerating axon would have in travelling the distance to its intended
target site and in making a functional synapse on its correct target while ignoring all the incorrect potential targets.

In contrast to the difficulty posed by long range axonal regeneration, there are forms of neuroplasticity that normally occur post-CNS trauma for which appropriate targeting may be less problematic. Spontaneous re-wiring of limb somatotopic maps occurs in the somatosensory and motor cortices as well as within the brainstem post-neurotrauma [51-53]. Rat corticospinal tract axons cut in the thoracic dorsal funiculus sprout rostrally into the cervical spinal cord gray matter resulting in novel forelimb whisker and trunk activation evoked by hindlimb motor cortex stimulation [54]. After unilateral pyramidotomy, axons originating on the uninjured side of the corticospinal tract sprout and grow into the denervated side of the cord [55, 56]. Following a lesion of the dorsal corticospinal tract, a small proportion of ventral corticospinal fibers sprout into the denervated dorsal spinal cord [57]. Hindlimb corticospinal tract axons were found to sprout into the cervical gray matter and connect with long propriospinal neurons which bridged the lesion site and innervated lumbar spinal cord motorneurons, and electrophysiological stimulation of the hindlimb motor cortex revealed these circuits to be functional [58]. All of these are examples of naturally occurring axonal plasticity post SCI. The reduction in CSPG expression seen in Sox9 KO mice may improve these endogenous reactive sprouting mechanisms, allowing newly formed collateral sprouts from spared axons traversing the lesion to make functional connections on motor neuron targets in the same field as the original targets of the injured axons.

If the spinal cord injured Sox9 KO mice displayed increased neuroplasticity we reasoned that it would be most evident around deafferented lumbar motor neurons demonstrating reduced PNN CSPGs. Thus, we investigated synaptic plasticity by examining acute and chronic changes
in pre-synaptic markers in the ventral horn of the lumbar enlargement in control and *Sox9* KO mice after SCI. After SCI, synaptic inputs in the ventral horn are decreased acutely and then, over time some synaptic inputs return [59]. This return of synaptic inputs is attributed to neuroplasticity within the spinal cord. At 48 h post SCI, *Sox9* KO mice and controls had approximately the same number of pre-synaptic synaptophysin vesicle markers remaining, supporting our finding that *Sox9* KO does not result in sparing of axons. However, at 10 weeks post-SCI *Sox9* ablated mice displayed increased synaptophysin immunoreactivity as well as increased synaptophysin+ individual puncta.

The synaptic losses observed at 48 hours after injury are likely due to substantial reductions in glutamatergic and GABAergic inputs, like those occurring in autonomic circuits [60]. As the majority of inputs onto spinal motor neurons are either glutamatergic or GABAergic [61, 62], densities of VGLUT1+ and VGAT+ presynaptic vesicles have been used to reveal changes in synaptic input due to remodeling after injury [61] as they reflect the number of synapses on cell bodies [34, 63, 64]. We thus investigated VGLUT1 and VGAT pre-synaptic markers in the *Sox9* KO mice. At 48 h post SCI, *Sox9* KO mice and controls had approximately the same number of VGLUT1+ and VGAT+ pre-synaptic vesicle markers remaining but by 10 weeks post-SCI *Sox9* ablated mice displayed increased VGLUT1 immunoreactivity as well as VGLUT1+ individual puncta compared to controls, and similar levels of VGAT immunoreactivity and individual puncta compared to controls. Thus following SCI the VGLUT1 pre-synaptic marker increases more quickly in the *Sox9* KO mice than in controls.

The increased presynaptic markers displayed in *Sox9* KO lumbar enlargements could indicate an increased number of neuronal synapses, an increased number of neurotransmitter release sites, or an increased number of presynaptic vesicles per release site. Each of these
possibilities are examples of neuroplasticity, and each would indicate increased synaptic strength in the lumbar enlargements of Sox9 KO mice 10 weeks post-SCI. As Sox9 KO mice did not display evidence of long-range axonal regeneration, this increase in presynaptic markers is likely due to the sprouting of spared fibers below the spinal lesion.

To investigate reactive sprouting in Sox9 KO mice we carried out an anterograde labeling study. Anterograde labeling demonstrated an increased number of BDA+ puncta around ventral horn motor neurons below the lesion in Sox9 KO mice in comparison to controls. Since we did not observe an increase in neuronal sparing in the Sox9 KO mice the increased BDA labelling indicates that fibers spared from injury undergo reactive sprouting to a greater extent in Sox9 KO mice than in controls. Our data also demonstrated that more anterogradely labeled pre-synaptic terminals co-localize with the VGLUT1 pre-synaptic marker in Sox9 KO mice than in control mice. This suggests that many of the reactive sprouts found in Sox9 KO mice terminate in VGLUT1+ pre-synaptic boutons near motor neurons, and are likely to be at least partly responsible for the improved functional recovery seen in these mice.

Others have shown that the limited recovery after SCI is likely due to remodeling of existing circuits rather than to the re-growth of descending supraspinal inputs [58, 65]. Thus we were not surprised to see an increase in reactive sprouting as opposed to long-range regeneration of damaged descending spinal tracts. As we did not find increased BDA+ puncta or VGLUT1+ puncta around ventral horn motor neurons in the cervical enlargement, we suggest that the increased reactive sprouting in Sox9 KO mice occurs due to two signals: deafferentation of target neurons and reduced levels of CSPGs in the PNNs of those target neurons.

Most spinal cord injuries in humans are in fact incomplete injuries where a proportion of axonal projections running through the lesion site remain intact post injury [66]. Thus, a
therapeutic strategy that promotes sprouting of these surviving spared axons may greatly improve functional outcome post SCI. It has been suggested that increased collateral sprouting in cervical level injuries might increase spared lower cervical level fiber innervation and improve control of the triceps, thus affording improved control over a wheelchair [67], and that increased innervation by spared serotonergic fibers might yield improved bladder control [68] or reduced pain [69].

We have previously shown that Sox9 ablation both before SCI and in the subacute period after SCI results in reduced CSPG levels in the spinal cord and improvements in motor function post-SCI. The present study extends our previous work by demonstrating that Sox9 ablation leads to increased reactive sprouting caudal to the lesion post-SCI. Taken together, increasing neuroplasticity by way of SOX9 inhibition is a promising therapeutic strategy for the treatment of SCI.
Supplementary Figure 1. Sox9 KO mice also do not display increased sparing or long range axonal regeneration in propriospinal interneurons, the rubrospinal tract or the vestibular spinal tract post-SCI. Uninjured mice display more fluorogold labeled propriospinal interneurons, rubrospinal tract neurons or vestibular spinal tract neurons, than in injured mice. Control and Sox9 KO mice do not display differing fluorogold labeling at 1 week post-SCI indicating that Sox9 KO does not improve sparing post-SCI in these tracts. Control and Sox9 KO mice do not display differing fluorogold labeling at 8 weeks post-SCI indicating that Sox9 KO does not result in increased long range (at least 1 spinal segment) axonal regeneration post-SCI in these tracts. (p=<0.05, one way ANOVA. * indicates significantly different from the injured groups.  a, b, c indicates significantly different from each other). Scale bars = 100μm.
Supplementary Figure 1. Sox9 KO mice also do not display increased sparing or long range axonal regeneration in propriospinal interneurons, the rubrospinal tract or the vestibular spinal tract post-SCI.
Supplementary Figure 2. Control and Sox9 KO mice display similar numbers of BDA labeled fibers in the cervical enlargement in both uninjured mice as well as 10 weeks post-SCI. A) BDA labeling in the cervical enlargement of uninjured control mice. B) BDA labeling in the cervical enlargement of uninjured Sox9 KO mice. C) The cervical enlargements in uninjured Sox9 KO and control mice display equal BDA labeling (p=>0.05, Student’s t-test). D) BDA labeling in the cervical enlargement of control mice post-SCI. E) BDA labeling in the cervical enlargement of Sox9 KO post-SCI. F) In the cervical enlargement (rostral to the injury site) Sox9 KO and control mice display equal BDA labeling (p=>0.05, Student’s t-test). Scale bars = 100μm.
Supplementary Figure 2. Control and Sox9 KO mice display similar numbers of BDA labeled fibers in the cervical enlargement in both uninjured mice as well as 10 weeks post-SCI.

Cervical enlargement in uninjured mice

Cervical enlargement in injured mice
Supplementary Figure 3. High magnification images display similar numbers of BDA and VGLUT1 positive puncta around ventral horn motor neurons in the cervical enlargement in Sox9 KO mice. Motor neurons in the ventral horn of the cervical enlargement stained for BDA+ puncta, VGLUT1+ puncta, and BDA-VGLUT1 co-localized puncta viewed by confocal z-stack. BDA confocal z-stack (A) and Imaris identified individual BDA+ puncta (B) in control mice. BDA confocal z-stack (C) and Imaris identified individual BDA+ puncta (D) in Sox9 KO mice. E) Motor neurons in the ventral horn of the lumbar enlargement displayed similar numbers of BDA+ puncta in control and Sox9 KO mice (p=<0.05, Student’s t-test). VGLUT1 confocal z-stack (F) and Imaris identified individual VGLUT1+ puncta (G) in control mice. VGLUT1 confocal z-stack (H) and Imaris identified individual VGLUT1+ puncta (I) in Sox9 KO mice. J) Motor neurons in the ventral horn of the lumbar enlargement displayed similar numbers of VGLUT1+ puncta in control and Sox9 KO mice (p=<0.05, Student’s t-test). NeuN (blue) BDA-VGLUT1 confocal z-stack (K) and Imaris identified individual BDA-VGLUT1+ co-localized puncta (L) in control mice. NeuN (blue) BDA-VGLUT1 confocal z-stack (M) and Imaris identified individual BDA-VGLUT1+ co-localized puncta (N) in Sox9 KO mice. O) Motor neurons in the ventral horn of the lumbar enlargement displayed similar numbers of BDA-VGLUT1 co-localized puncta in control and Sox9 KO mice (p=<0.05, Student’s t-test). Scale bars indicate 10µm.
Supplementary Figure 3. High magnification images display similar numbers of BDA and VGLUT1 positive puncta around ventral horn motor neurons in the cervical enlargement in Sox9 KO mice.
4.5 Acknowledgments

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4.6 Author Disclosure Statement

A.B. holds a patent on SOX9 inhibition as a target for regeneration in the nervous system.

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4.7 References


Chapter 5: Discussion

5.0 Sox9 knockdown, a potential pro-regenerative treatment for SCI

The ultimate goal of post-SCI nervous system repair strategies is to reestablish the required neuronal connections for recovery of neurological function. Such connections may occur by way of retracted axons undergoing long-distance regeneration through the lesion site followed by proper synapse formation on downstream motor neurons. For this type of regeneration to occur, damaged axons would need not only to survive near the post-injury lesion epicenter, but would also need to extend through the inhibitory lesion site to the caudal spinal cord and make functional synapses on correct targets while choosing from millions of potentially incorrect targets. Although there have been no confirmed reports of such regeneration in humans, there is reason to believe pro-regenerative treatments for chronic SCI can be developed.

The initiation of walking is handled by the brain that sends a message down spinal cord axons to initiate walking [1]. This message needs to be received by a center in the lumbar spinal cord referred to as the central pattern generator (CPG) that initiates and coordinates the muscles required for proper walking motion [2]. The CPG region contains neural circuits that produce self-sustaining patterns of muscle activation independently of sensory input [3]. CPG activity has been observed in newborn children including anencephalic newborns suggesting that CPGs are likely to exist in adult humans as well [4, 5]. It is possible to exogenously stimulate the lumbar enlargement in the lower spinal cord and activate a walking motion [2, 6]. In humans with significantly impaired ability to move their legs, epidural spinal cord stimulation of lumbar segment 2 results in improved locomotion as well as recruitment of more muscle fibers and
improved gait post incomplete SCI [7-9]. A significant challenge then is to ensure the brain can send the required initiation of walking signal through the spinal cord to this central pattern generator machinery. While considering this, it is important to note that rats and cats can walk normally with less than 10% of their spinal cord intact at a lesion site [10], and spinal surgeons have suggested that this may be true for humans as well [11]. If only 10% of the axons in the spinal cord are necessary and sufficient to initiate locomotor activity, it is not unreasonable to think pro-regenerative treatments for chronic SCI could be developed. In this thesis I investigated one such potential pro-regenerative treatment, the effect of Sox9 knockdown on hind limb function and neuroplasticity post-SCI in the mouse.

5.1 Obstacles to regeneration, activation of SOX9 post-SCI

Many animals found low on the evolutionary tree such as teleost fish, the salamander, and newt display profound axonal regeneration throughout life whereas more evolved animals such as mammals do not [12]. In fact, the CNS of mammals develops early in ontogeny, and once the critical period of plasticity closes in early adulthood mammals only display regeneration of their PNS [12, 13]. Thus, it may be more feasible to reprogram the local environment of an adult mammal to be more reminiscent of early life so as to activate pro-regenerative genetic programs native to the CNS as opposed to trying to activate regenerative programs native to the PNS or those found in more primitive animals. Unfortunately there are significant cellular and molecular differences between the adult-injured spinal cord and the embryonic spinal cord [14], not the least of which being that the development of the spinal cord takes place in an environment devoid of inflammation, and that the inflammatory response is dramatically up-
regulated post-SCI resulting in a complete change in the local microenvironment. One of the key outcomes of increased inflammation is a cytokine and chemokine storm that contains many molecules capable of activating the transcription factor SOX9. Pro-inflammatory cytokines including transforming growth factors β1 and 3, interleukin-6, and platelet-derived growth factor all work to activate SOX9 [15, 16]. Thus the inflammatory environment post-SCI provides ample opportunity for SOX9 activation, increased glial scarring, and amplified expression of anti-regenerative CSPGs. We hypothesized that inhibition of SOX9 would combat these environmental changes.

### 5.2 Sox9 knockdown results in improved hind limb motor function post-SCI

Our contusion model of SCI results in paralysis of the hind limbs immediately post-injury. Injured mice recover a minimal degree of hind limb function over time. In our initial proof of principle study we knocked down SOX9 expression in a group of mice prior to SCI. These Sox9 knockdown mice displayed remarkably quick recovery of hind limb function post contusion, scoring statistically higher than control mice expressing normal levels of SOX9 on the Basso Mouse Scale by 1 week post-SCI. This statistically significant improvement continued throughout the 14 weeks of our initial proof of principle study. The obvious caveat to this study was that the experiment occurred in mice which had already had Sox9 knocked out prior to SCI. This type of experimental design works well to demonstrate the potential of a SOX9 inhibition based therapy, but does not speak toward the clinical feasibility of such a treatment. To address this we conducted a follow up study where our contusion injury occurred 1 week prior to tamoxifen administration. As it requires roughly 6 days of tamoxifen administration for
appreciable *Sox9* knockdown to occur this second study investigated the effect of *Sox9* knockdown almost two weeks post-SCI. In this model *Sox9* knockdown mice performed statistically better than control mice on the Basso Mouse Scale from 6 weeks post-SCI. This study demonstrates not only a clinical relevance and potential of an anti-SOX9 treatment for SCI, but suggests that the mechanism leading to recovery seems to be effective even after glial scar formation in *Sox9* knockdown mice. We next investigated the anatomic mechanism behind this functional improvement.

**5.3 *Sox9* knockdown reduces anti-regenerative CSPG expression post-SCI**

Following SCI damaged axons retreat a short distance before they attempt to grow back towards the injury site. Contact with CSPGs rich inhibitory extracellular matrix throughout the lesion site leads to growth cone collapse and axonal dystrophy, however these dystrophic axons are surprisingly dynamic and continually attempt to cross the lesion site [17]. Some invade the lesion area but do not exit on the distal side, but the great majority stop at the border of injury and appear to wait there even years post-SCI [18, 19]. If provided an avenue for growth through the inhibitory extracellular matrix, spinal axons do possess the ability to not only grow in the spinal cord but to form functional synapses on novel targets. David & Aguayo used peripheral nerves to bridge a spinal lesion and found spinal axons would grow into and through these nerves [20, 21]. These axons were found to innervate muscle at the distal end of the inserted peripheral nerve [22], and form functional glutamatergic synapses on their new targets [23, 24]. These studies emphasized how the inhibitory matrix in the local tissue microenvironment plays a key role in regulating axonal growth post-SCI [25]. It is also pertinent to note that lampreys, teleost
fish, and the urodele amphibians that can regenerate an injured spinal cord throughout their lifespan do not develop a CSPG rich glial scar [26]. Sox9 knockdown mice consistently display a reduction in CSPG expression both at the mRNA and protein level at the lesion epicenter and in the lesion penumbra. Our prediction was that the reduction in inhibitory matrix in Sox9 knockdown mice would provide a needed avenue for growth through the glial scar and allow dystrophic axons to finally traverse the lesion. Sox9 knockdown mice also display reduced PNN matrix throughout the lesion site, and at the distant lumbar enlargement. Enzymatic digestion of PNNs by chondroitinase results in reactivation of plasticity [27, 28], and enhances spared fiber reactive sprouting and synapse plasticity [29], in injured adult animals. We therefore investigated whether the Sox9 knockdown mice displayed increased neuroplasticity that could account for their improved hind limb motor function following SCI.

5.4 Investigating neuroplasticity post-SCI; axonal regeneration, reactive sprouting, and synapse plasticity

There are three main types neuroplasticity leading to recovery post injury; axonal regeneration, axonal sprouting, and synaptic plasticity. Axonal regeneration refers to the growth of an injured axon over long distances across, and extending beyond, the lesion site [30, 31]. Successful regeneration would thus require a damaged axonal fiber to grow several millimeters or more along its original trajectory toward its original synaptic partner [30]. Axonal sprouting applies to growth from either a damaged or intact axonal fiber over shorter distances [30, 31]. There is no precise definition as to the required length at which axonal growth transitions from sprouting to regeneration [30]. Reactive sprouting occurs in response to the injury when an uninjured fiber sprouts and makes new connections in an attempt to re-connect damaged tracts
Synaptic plasticity is the re-organization of existing synapses, or the creation of new synapses which would result in correct activation of the original targets which lost connectivity as a result of SCI [32].

Due to reduced glial scar CSPGs and lumbar enlargement PNNs, Sox9 knockdown CNS axons damaged at the time of SCI should have a more hospitable growth environment through the lesion, and the lumbar enlargement environment should be more permissive to reactive sprouts finding motor neuron targets. We thus predicted that increased long range regeneration would occur through the glial scar, and that increased reactive sprouting and increased synaptic plasticity would occur in the ventral horn of the lumbar enlargement.

5.5 *Sox9* knockdown does not promote axonal sparing post-SCI

Prior to assessing axonal plasticity we must ensure that we rule out the possibility of axonal sparing in the *Sox9* knockdown mice so as to not confuse increased axonal sparing with increased axonal growth. We saw no evidence of descending axonal sparing in *Sox9* knockdown mice. We observed similar amounts of neurofilament traversing the lesion site in *Sox9* knockdown and control mice post-SCI. Our 1 week post-SCI fluorogold labeling study confirmed the absence of increased sparing in the *Sox9* knockdown mice by demonstrating equal numbers of retrogradely labeled neurons post-SCI in *Sox9* knockdown and control mice. Thus, *Sox9* knockdown mice do not display increased axonal sparing post-SCI. As the *Sox9* knockdown mice did not display evidence of axonal sparing, but did display reduced anti-regenerative extracellular matrix, we turned our attention to an investigation of axonal plasticity.
5.6 *Sox9* knockdown does not promote long range axonal regeneration post-SCI

Although we hypothesized that successful reduction in CSPG expression throughout the lesion environment would result in improved long range axonal regeneration, we saw no evidence of this. The lesion epicenter in injured *Sox9* knockdown mice 14 weeks post-SCI did not display increased neurofilament immunoreactivity arguing against significant regeneration across the lesion site. Secondly, our retrograde labeling study demonstrated a significant loss of labeled axonal tracts 1 week post-SCI, and that none of the injured tracts studied displayed long range axonal regeneration 8 weeks post-SCI.

The *Sox9* knockdown mice do however display improved locomotor function in comparison to controls post-SCI. Long range regeneration may not be occurring in our *Sox9* knockdown mice, but there are other potential mechanisms for regeneration known to occur endogenously post-SCI that are based on smaller neuronal changes that could potentially be improved by *Sox9* knockdown. Thus, we investigated short range reactive sprouting of spared axons as well as axonal synapse plasticity.

5.7 *Sox9* knockdown results in increased short range reactive sprouting and synapse plasticity post-SCI

An endogenously occurring compensatory and functional response to denervation post-SCI, termed reactive sprouting, has been studied for over 50 years. In 1958 McCouch et al. demonstrated that sensory afferents undergo sprouting in the spinal cord after SCI in both cats
and monkeys [33]. CSPGs restrict this reactive sprouting as chondroitinase injection into the brainstem post-cervical SCI resulted in digestion of PNNs and anatomical evidence of sprouting by spinal cord afferents in the cuneate nucleus [34]. Further studies on combined BDNF and chondroitinase treatment induced significant sprouting of undamaged retinal afferents into the denervated superior colliculus after a partial retinal lesion in the adult rat [29], and anti-synapsin antibody staining demonstrated increased synapse plasticity at the ends of the newly sprouted axons [29]. Beneficial alterations to the strength of existing neural connections may also occur post-SCI. An increased density of postsynaptic neurotransmitter receptors, increased excitatory neurotransmitter release, decreased inhibitory neurotransmitter release, or the removal of inhibition from excitatory input would all be beneficial to recovery from SCI [35, 36]. It is these types of short range plasticity we believe Sox9 knockdown facilitates, and are in fact much easier to imagine occurring in a human than the aforementioned long range regeneration.

Recovery in the Sox9 knockdown mice occurred quickly, within 1 week in the proof of principle model. It would thus seem unlikely that long range regeneration would be the mechanism behind such quick recovery, and therefore it was not surprising that we did not see evidence of long range regeneration in the Sox9 knockdown mice. Short-range sprouting of spared axons onto deafferented targets due to the profound reduction in PNN burden in the Sox9 knockdown mice would be expected to occur rapidly. This type of repair may also be less prone to mis-wiring of circuits as the most likely axons to form new synapses on a target will be those that are already innervating adjacent neurons in the same target field. We hypothesized that PNN reduction in the distant lumbar enlargement would allow for increased reactive sprouting and synapse plasticity in the ventral horn of the lumbar enlargement.
Our neurofilament immunostaining suggests the same amount of axonal matter crosses the lesion site in both controls and $Sox9$ knockdown mice, however significantly more neurofilament was noted just rostral and just caudal to the injury penumbra. This suggests local sprouting of spared axons and potentially bridging connections created to circumvent the lesion site. Our BDA labeling data confirm these hypotheses as significantly increased BDA labeling was noted in the distant lumbar enlargement. As more BDA positive puncta were found in the lumbar enlargement, and PNNs in the lumbar enlargement were found to be down regulated in $Sox9$ knockdown mice we were not surprised to see increased synaptic plasticity in these animals’ lumbar enlargements. We noted increased synaptophysin expression in the ventral horn of $Sox9$ knockdown mice lumbar enlargements. Synaptophysin was increased not only in total immunostaining, but also in the number of individual synaptophysin+ puncta, suggesting either increased synaptic release sites, increased vesicles per release site, or increased number of individual synapses. Each of these possibilities would indicate increased synaptic plasticity in these animals. Our data thus suggest that spared descending supraspinal and/or propriospinal axons sprout beneath the lesion and form synapses on ventral horn layer VIII and IX targets, probably motor neurons. We also investigated excitatory and inhibitory input in the ventral horn by assessing vesicular glutamate transporter VGLUT1 (excitatory neurotransmitter of the corticospinal tract) and vesicular GABA transporter VGAT (inhibitory neurotransmitter of the corticospinal tract) neurotransmitter expression [37]. We found increased VGLUT1 excitatory input and unchanged VGAT inhibitory input in $Sox9$ knockdown mice in comparison to controls post-SCI.

The majority of human SCI are incomplete [19], and it has been suggested that these incomplete SCI may be more permissive to regeneration as cortical and subcortical structures, as
well as distant spinal cord circuitry, remain largely intact, while the damaged local spinal cord circuitry still remains partially connected by unlesioned axonal fibers [32]. Reactive sprouting as described herein would fit this model as a potential mechanism to stimulate beneficial neuroplasticity in the human post-SCI.

It is important to note that untargeted sprouting might lead to abnormally formed reflexes and detrimental sprouting of uninjured sensory afferents. However, the BDA labeling experiment described herein found no evidence for sprouting in the cervical enlargement away from the injury site. *Sox9* knockdown mice did not display altered numbers of BDA fibers identified in the cervical enlargement. We also investigated BDA labeling as well as VGLUT1 expression around ventral horn motor neurons in the cervical enlargement, but did not find increased BDA positive puncta or VGLUT1 staining. Thus, we suggest that the neuroplasticity displayed by *Sox9* knockdown mice described herein requires not only a decrease in anti-regenerative CSPG and PNN expression, but also the loss of synaptic input and creation of free synaptic space. Given our T9 SCI model, it would be expected that motor neurons of the lumbar enlargement would display significant denervation whereas those in the cervical cord would likely be undamaged. This lack of denervation in the cervical enlargement may not have allowed the reactive sprouting or synaptic plasticity demonstrated in the lumbar enlargement. Thus, it is possible that the great majority of functional connections that are made due to increased plasticity after reduction of CSPG expression occur near the injury site. They likely serve as positive forms of neuroplasticity allowing restored functionality rather than detrimental improperly formed connections. Adding credence to the belief is data from chondroitinase treated animals suggesting that, despite the increased growth, sprouting and connectivity displayed following chondroitinase treatment there has been no reported evidence of increased sensitivity to pain in
these animals [38-40]. However, previous studies have demonstrated that, following a hemisection at T13 and a complete transection at T5, sensory afferents sprouted in the dorsal horn and are believed to have contributed to the development of mechanical allodynia and autonomic dysreflexia [41, 42]. Although we see no evidence of such changes through day to day handling and visual observation of Sox9 knockdown mice, a thorough investigation of pain and sensory function is an important study that needs to be conducted. The presence of mechanical allodynia (a pain syndrome in which innocuous stimuli are perceived as painful) could be assessed by stimulating the backs of injured mice with a Semmes Weinstein monofilament at, and rostral, to the level of SCI. The number of avoidance responses (attempts to escape, vocalization, jumping, flinching and/or attempting to bite the filament) due to the stimulation over a number of trials would be recorded and compared between control and Sox9 knockdown mice. Autonomic function could be assessed by measuring autonomic dysreflexia (episodic hypertension triggered by sensory stimulation below the level of the spinal lesion) in the mice. Autonomic dysreflexia is thought to be due to the loss of descending inhibitory inputs and the generation of abnormal reflexes in the injured spinal cord [43]. Autonomic dysreflexia can be assessed by measuring increases in blood pressure in response to colon distension [44], the extent of which correlates with the degree of SCI [45]. Blood pressure increases due to the stimulation over a number of trials could be recorded and compared between control and Sox9 knockdown mice.
5.8 Combination therapies may be required to produce maximal beneficial effect post-SCI

The reduction in the inhibitory CSPG barrier which results from SOX9 inhibition creates an environment permissive to neuroplasticity, but optimal axonal regeneration may require exogenous administration of other growth factors, and perhaps inhibition of other anti-growth factors. Molecularly, the signaling pathways which regulate neurite and axonal growth are complex. Addition of neurotrophins [46], and inhibition of myelin-associated inhibitory molecules such as Nogo, myelin associated glycoprotein or myelin oligodendrocyte glycoprotein, may prove beneficial in promotion of reactive sprouting. For a review see Hagg et al. [47]. Recent studies suggest that combining pharmacological and activity based therapies may yield the best results post-SCI. Chondroitinase administration in concert with activity training resulted in improved rat forelimb function and locomotion recovery post-SCI [48]. Rats were assigned to one of two rehabilitation paradigms, the first receiving only skilled reaching training, and the second receiving only locomotion training. Chondroitinase treatment resulted in increased sprouting of corticospinal axons in both rehabilitation groups. Rats receiving the combination of chondroitinase and reaching training displayed increased reaching ability. Rats that received chondroitinase and locomotor training displayed improved locomotor abilities. Importantly, a major caveat appeared in this study. The rats that underwent the locomotor training scored worse on reaching tests than rats that received no specific activity training. The authors conclude that, although reducing CSPG burden opens a window of plasticity during which rehabilitation can promote recovery, only specifically trained skills appeared to improve, and other functions may in fact be negatively affected. Thus, combination therapies that emphasize a particular type of activity may eventually prove beneficial, but significant work needs to be undertaken to characterize the full effect of activity training on neuroplasticity and
recovery post-SCI. Regardless, we recognize that in the future combined therapies that target more than one axon-repelling molecule or make use of physical training may yield the best results post-SCI.

5.9 Complete Sox9 knockdown is unlikely to be optimal for recovery post-SCI; beneficial role of astrocytes post-SCI

One of the benefits of a SOX9 targeted approach is the coinciding reduction in astrocyte activation (GFAP expression). It is however important to note that it is likely unwise to completely eliminate astrocyte activation and glial scarring post-SCI. Thus close regulation of SOX9 activity will be required in any future anti-SOX9 therapy devised for treatment of SCI.

Astrocytes do not only express molecules known to block axonal regeneration, but have also been shown to secrete extracellular matrix molecules conducive to axonal growth such as laminin, N-cadherin [49], neural cell adhesion molecule [50], and fibronectin [51]. Laminin and fibronectin have been shown to be good substrates for neurite extension in various in vitro models [52]. In neuron-astrocyte co-cultures, the ability of astrocytes to support neurite outgrowth depends upon astrocyte expression of laminin [53]. Neonatal rats regenerate transected connections and recover locomotor function after SCI [54], perhaps because glial tissue from lesioned neonates is rich in laminin and fibronectin and adequately supports neurite extension [55]. In general, astocyte cell lines that support neurite outgrowth produce an ECM rich in laminin and fibronectin while astrocyte lines producing ECM rich in CSPGs are nonpermissive to neurite extension [56]. In vivo, the regeneration of microtransplanted sensory neurons through inhibitory CNS myelin has been shown to be dependent on astrocyte-associated
fibronectin [51, 57, 58] and intrathecal administration of laminin promoted regeneration in a rat model of SCI [59]. Astrocytes provide trophic support including required metabolites, nutrients, and growth factors, brain-derived neurotrophic factor and neurotrophin 3, to the injury site which may help preserve spared neurons [60, 61]. Astrocytes play a key role in molecular scavenging and are thus important in regulating excessive levels of glutamate, potassium, and other ions post-SCI. In fact, astrocytes can directly protect neurons from nitric oxide toxicity through a glutathione-dependent mechanism [62]. The glial scar itself may be crucial for the survival of spared axons as it helps create a scaffold for the vasculature. Astrocytes and extracellular matrix components recruit endothelial cells and fibroblasts to the lesion site and induce the formation of new capillaries [63]. Recent studies suggest that complete removal of astrocytes results in significantly larger lesions, local tissue disruption, demyelination, and local neuron and oligodendrocyte death [64-67]. Thus, it is not optimal to completely inhibit astrocyte activation post-SCI.

5.10 Complete Sox9 knockdown is unlikely to be optimal for recovery post-SCI; beneficial role of CSPGs post-SCI

It is also important to consider the possibility that CSPGs may be physiologically necessary to minimize inflammation and provide a scaffold for remodeling post-SCI [68, 69]. Thus, completely ablating CSPG presence at the site of injury may in fact exacerbate SCI rather than promote recovery. Michal Schwartz’s group has shown that inhibition of CSPG expression immediately following SCI results in increased leukocyte infiltration, a dramatic change in microglia/macrophage organization at the lesion, decreased growth promoting insulin-like
growth factor 1 production, and increased pro-inflammatory tumor necrosis factor alpha production, by microglia/macrophages. This contributed to impaired functional motor recovery, and increased tissue loss. Inhibiting CSPG expression 2 days post-injury resulted in significantly improved outcome [70]. Their conclusion was that, in the acute stages of SCI, CSPGs appear to be beneficial for repair and subsequent recovery of function, but that chronic CSPG production does in fact inhibit axonal regeneration [70]. Thus, maximal recovery and improvements in quality of life for those with a nervous system injury may require careful modulation of CSPG levels over time.

5.11 Why do we our Sox9 knockdown mice only display ~65% Sox9 reduction?

Given that astrocytes and CSPGs undoubtedly make some beneficial contribution to wound healing and recovery post-SCI, it is likely wise to allow a moderate degree of astrocyte activation and CSPG production post-SCI. Thus, our conditional knockdown strategy may have significant advantages over a complete knockout model. As we only see ~65% Sox9 knockdown following tamoxifen administration a significant amount of SOX9 remains to carry out its normal function. With this said, it is interesting to contemplate why we have not seen more complete Sox9 knockdown post tamoxifen administration. In the animals used for these experiments Sox9 knockdown occurs due to Cre recombinase acting on loxP sites flanking exons 2 and 3 of the Sox9 gene. Researchers in the Brown laboratory have investigated Cre recombinase activity in ROSA-YFP mice in which tamoxifen administration activates YFP expression by removing a STOP codon in front of the YFP gene. As both the Sox9 recombination, and the ROSA-YFP recombination, events are based on Cre activity, they should
occur equally under the same tamoxifen administration schedule. However, in practice these recombination events do not occur with equal frequencies. While we see ~65% Sox9 knockdown, we see ~90% YFP expression as assessed by immunohistochemistry and flow cytometry. In a neural stem cell-specific Sox9 knockdown mouse model that also contains the ROSA-YFP allele ~20% of the YFP positive cells found in the ependymal layer (those that were affected by tamoxifen and had a recombination event) still expressed SOX9 (unpublished observation in the Brown Laboratory). This indicates that when a cell receives tamoxifen the Cre enzyme is more efficient at excising the STOP codon in front of the ROSA-YFP than the Sox9 loxP flanked locus. In the mouse the ROSA locus resides on chromosome 6, and the Sox9 locus resides on chromosome 11 (Genbank). We hypothesize that the Cre recombinase has easier access to the loxP sites flanking the STOP codon preceding the ROSA locus than to the loxP sites flanking Sox9. Such a situation would account for the reduced Cre recombination efficiency seen in Sox9 knockdown mice in comparison to the ROSA-YFP mice.

5.12 Alternative competing strategies, chondroitinase ABC

Chondroitinase is a very promising strategy currently being investigated for treatment of human SCI. There are however significant caveats with chondroitinase treatment which must be addressed. First, there are inherent dangers associated with treating humans with bacterial enzymes due to the potential for eliciting a damaging immune response. Chondroitinase also loses its enzymatic activity at body temperature [71] and must be repeatedly administered by intrathecal injection or infusion, an administration strategy that is not only invasive but also prone to inducing infection. Chondroitinase also does not diffuse far within the cord and thus
only acts on CSPGs at the local injection site. Finally, chondroitinase attempts to cleave carbohydrate stubs off of the large CSPG core proteins, but small inhibitory stubs remain as do the large core proteins themselves, thus axonal regeneration and functional recovery is limited [72, 73].

5.13 Alternative competing strategies, decorin

Another strategy being investigated is the use of decorin, a small proteoglycan with high affinity for binding transforming growth factor β. This interaction prevents transforming growth factor β from interacting with its receptors and thereby inhibits its signaling. As transforming growth factor β is a key inducer of CSPG biosynthesis, decorin has been used to inhibit glial scarring at the site of injury. Significant reduction of inhibitory proteoglycans neurocan, NG2, phosphacan and brevican was seen following pump infusion of recombinant decorin into acute stab injuries in the adult rat spinal cord [74]. Decorin also allowed transplanted adult sensory axons to grow across the acute stab injuries [74]. Decorin treatment resulted in an increase in plasminogen and plasmin protein expression, molecules which cleave CSPGs and activate neurotrophins and thereby seem a likely candidate for promoting axonal plasticity post-SCI. However, until now no reports have suggested that decorin confers a functional benefit post-SCI.

5.14 Alternative competing strategies, inhibition of N-acetylgalactosaminyltransferase-1

A third strategy aims to reduce CSPG expression while increasing heparan sulphate proteoglycan (HSPG) expression. HSPGs are structurally similar to CSPGs, but have been
shown to promote axonal growth. The assembly of HSPGs and CSPGs rely on the same initial enzymatic activity by XT-I and XT-II, thus by inhibiting XT-I and XT-II activity by way of Sox9 knockdown we may be inhibiting not only CSPG synthesis but HSPG synthesis as well. An interesting competing strategy to the one presented herein made use of inhibitors to the downstream enzyme N-acetylgalactosaminyltransferase-1 which will specifically block CSPG synthesis but allow HSPG synthesis. Following SCI, N-acetylgalactosaminyltransferase-1 knockdown mice display significantly increased functional recovery [75]. As Sox9 knockdown mice appear to display a reduced scarring phenotype in general, it is possible that Sox9 knockdown mice may display reductions in N-acetylgalactosaminyltransferase-1 post-SCI. As Sox9 knockdown does not completely eliminate XT-I and XT-II expression it is still possible that HSPGs are being synthesized. Therefore, it would be interesting to investigate N-acetylgalactosaminyltransferase-1 and HSPG expression in Sox9 knockdown mice.

5.15 Alternative competing strategies, inhibition of Nogo-A

Nogo-A is a neurite growth inhibitory molecule expressed by differentiated oligodendrocytes and found in high concentrations in CNS myelin [76]. The interaction between myelin based Nogo-A and the Nogo-66 receptor on the axon surface restricts neuronal growth through negative modulation of growth cone actin dynamics [77]. Following SCI in the rat, inhibiting Nogo-A with an antibody resulted in improved CNS axon regeneration [78], and inhibiting the Nogo-66 receptor with a competitive antagonist resulted in significant corticospinal tract regeneration, and improved functional recovery [79]. Targeting Nogo-A has proven beneficial in primates as well as the recovery of manual dexterity and sprouting of
corticospinal tract axons were improved post-SCI in monkeys treated with anti-\textit{Nogo-A} antibody [80, 81].

Early phase clinical trials targeting \textit{Nogo-A} are currently in progress for the treatment of SCI. The pharmaceutical company Novartis AG created a humanized anti-\textit{Nogo-A} antibody which has been used for a Phase I clinical trial. The antibody was delivered intrathecally via a pump for time periods ranging from 24 h to 4 weeks. The drug was tolerated well with no reported side effects. A Phase II study is currently underway and aims to test the efficacy of the anti-\textit{Nogo-A} antibody treatment in paraplegic and quadriplegic SCI patients [82]. The authors admit that dosing will be particularly challenging as completely inhibiting \textit{Nogo-A} activity is in fact detrimental as seen in \textit{Nogo-A} KO mice which do not display increased neuronal growth or recovery due to a compensatory up-regulation of other inhibitory factors such as \textit{ephrinA3}, \textit{ephrinA4}, \textit{Sema 4D} and \textit{3F} and \textit{plexin B2} [83].

\textbf{5.16 Alternative competing strategies, inhibition of \textit{Rho}}

Another potential treatment for SCI focuses on the inhibition of the small GTPase \textit{Rho}. Following SCI there is an up-regulation of \textit{Rho} activity due to the release of growth inhibitory proteins and inflammatory cytokines whose receptors signal to \textit{Rho} [84]. Activated \textit{Rho} inhibits axonal growth by acting on the actin cytoskeleton and thus collapsing the neuronal growth cone [85, 86]. \textit{Rho} normally cycles between the cytosolic compartment and the cell membrane where it interacts with its receptors. \textit{Clostridium botulinum} exoenzyme C3 (C3) leads to sequestration of \textit{Rho} in the cytosolic compartment, preventing its interaction with receptors on the cell
membrane [87]. Local application of C3 to the injury site in a mouse hemi-section model of SCI resulted in long-distance regeneration of cortico-spinal neurons, and improved functional recovery as assessed by locomotor scores and limb coordination [88, 89].

Cethrin is a cell-permeable derivative of Clostridium botulinum exoenzyme C3 (C3) which has been investigated in a Phase I/IIa clinical trial. In a group of thoracic or cervical SCI patients Cethrin was delivered as a gel topically to the spinal cord at the time of decompression surgery by placement on the dura mater of the spinal cord at the end of the surgical procedure, a delivery mechanism shown to be effective in rodents [84]. Motor recovery scores displayed a trend suggestive of neurological recovery [90]. However, the authors compared their motor outcomes to historical controls, thus a larger scale trial comparing Cethrin to placebo treatment will need to be conducted so as to properly assess efficacy. The authors also suggest increasing the Cethrin dosage for a follow up study. Of course, increased dosage would need to be closely monitored for safety.

5.17 Alternative explanations for functional recovery post Sox9 knockdown

All of the above strategies hold promise as potential future treatments for SCI. However, all of the above strategies also focus on combating anti-regenerative molecules up-regulated following SCI. There are several alternative explanations for the recovery seen in Sox9 knockdown mice that do not focus on combating anti-regenerative CSPG molecules. These alternative mechanisms have not as of yet been thoroughly studied, but may contribute to improved functional recovery post-SCI in Sox9 knockdown mice. Such versatility and the
potential to promote regeneration and recovery through multiple avenues may be the biggest advantage of an anti-SOX9 based therapy for SCI.

A reduction in CSPG expression leading to an environment more permissive to axonal regeneration is not the only potential route by which functional recovery could be occurring in Sox9 knockdown mice. Inflammation is one of the key causes of secondary SCI, and is at least partially the result of leukocyte recruitment by activated astrocytes, thus as a result of decreased astrocyte activation Sox9 knockdown might lead to reduced inflammation at the site of injury. SOX9 may also play a role in neural precursor cell differentiation down a glial lineage, thus Sox9 knockdown might lead to reduced gliogenesis and increased neurogenesis. It is probable that the functional recovery seen in Sox9 knockdown mice is due to a combination of these potentially contributing mechanisms.

5.18 Alternative explanations for functional recovery post Sox9 knockdown; the effect of Sox9 knockdown on inflammation

The inflammatory response is one of the major contributors to secondary injury after SCI. Following SCI, endothelial cells up-regulate vascular cell adhesion molecules and reactive astrocytes express cytokine pro-inflammatory and chemoattractant molecules which attract inflammatory leukocytes to extravasation from the blood to the injured tissue. Leukocytes recruited to the site of injury may play a phagocytic role cleansing the area of bacterial infection; however, such activity can be detrimental to the spinal cord. The release of oxygen free radicals and reactive nitrogen species during phagocytosis can lead to neuronal damage through demyelination and scarring of spinal tissue [91, 92]. In addition, the release of pro-inflammatory
cytokines from activated phagocytes promotes further inflammation. Ongoing damage to neurons and the area surrounding the primary lesion contributes to increased lesion size and further neurological dysfunction [92, 93]. Inhibiting the inflammatory response by macrophage depletion following SCI decreases cavitation size and improves axonal regeneration [93, 94].

Sox9 knockdown mice display reduced GFAP expression. However, we have noted similar levels of glutamine synthetase positive cells in Sox9 knockdown and control mice (unpublished observation). In the brain, glutamine synthetase is expressed predominantly in astrocytes. Thus Sox9 knockdown mice display the same numbers of astrocytes as measured by glutamine synthetase, but a reduced astrocyte activation state as indicated by reduced GFAP expression. Reduced astrocyte activation may translate into reduced pro-inflammatory chemokine and cytokine production by astrocytes, and thus Sox9 knockdown mice may display a muted inflammatory response to SCI.

A pilot study was conducted finding that in comparison to control mice Sox9 knockdown mice demonstrate reduced macrophage numbers at the lesion site 14 weeks post-SCI (unpublished observation). This opens an interesting avenue for the future investigation of the effect of Sox9 knockdown on the inflammatory response. As we have only investigated macrophage present at the lesion site at the end point of our study (14 weeks post-SCI), a complete time course of leukocyte infiltration must be conducted, as well as evaluation of reactive oxygen and nitrogen species, and determination of a complete pro-inflammatory cytokine profile at the lesion site post-SCI. If indeed anti-inflammatory, timing an anti-SOX9 based intervention would be critical as the inflammatory response operates in several phases, with an initial more destructive phase composed predominantly of neutrophil influx over the first 24 h post-SCI [95], followed by a pro-inflammatory phenotype macrophage influx between days
2 and 7 [95], and finally a more drawn out macrophage response over the second week of injury, thought to be beneficial for wound healing.

5.19 Alternative explanations for functional recovery post Sox9 knockdown; the effect of Sox9 knockdown on neural stem cells

Neural stem cells constantly self-renew and eventually differentiate into neurons, astrocytes, or oligodendrocytes [96]. Considerable research has been conducted investigating the transplantation of exogenous neural stem cells as a potential method to replace cells lost at the time of injury or to produce new neurons capable of building connections across the injury site and contributing to improved function [97]. One of the main findings of these works was that the majority of cells surviving the transplant differentiate into astrocytes and do not result in improved functional recovery [98, 99]. Perhaps the injury environment only supports glial neural stem cell differentiation. This is not surprising as neural stem cells differentiate into neurons when transplanted into hippocampal brain areas, but differentiate into astrocytes when transplanted into uninjured spinal cords [100]. It has been shown that by priming or genetically modifying neural stem cells it is possible to promote their neural differentiation once transplanted into the CNS [97, 101-104]. However, such strategies are not optimal clinical treatments as injecting highly proliferative cells may lead to tumor formation [105], and still rely on transplant allograft with a high likelihood of immunological rejection [106]. It may be safer and more efficient to alter the differentiation of the endogenous neural stem cells to produce more neurons and oligodendrocytes, and fewer astrocytes.
The developing CNS in Sox9 knockdown mice displays altered neuronal/astroglial numbers. Twelve and one half days post conception Sox9 deficient embryos displayed a 30% increase in motor neurons, and at 14.5 days post conception Sox9 knockdown embryos displayed 25% as many oligodendrocyte progenitors as wild-type embryos and almost a complete lack of astrocytes in their spinal cords [107]. Together these findings suggest that during development SOX9 promotes neural stem cell differentiation down the astroglial lineage while inhibiting differentiation into new neurons. SOX9 is also required to maintain fully functioning neural stem cells as modulation of SOX9 expression results in altered neurosphere formation [108]. In both mouse and chick embryos neural stem cells appear just after up-regulation of SOX9 expression. Increasing SOX9 expression at an earlier time point before the normal appearance of neural stem cells resulted in early neurosphere formation and when Sox9 was knocked out fewer neurospheres were found [108]. miRNA treatment has since confirmed the finding that inhibiting Sox9 expression reduces neural stem cell function and proliferation as well as the formation of astroglia, while promoting neuronal differentiation [109].

In the Sox9 knockdown mice described herein tamoxifen administration results in Sox9 ablation in all cell types. Thus the improved locomotor recovery seen could be the result of reduced SOX9 expression in a variety of cell types, with astrocytes and neural stem cells known to be the two major cell types in the spinal cord that express SOX9 [110]. The improved functional recovery seen in these mice may not be due to just reduced CSPG expression leading to increased neuroplasticity of existing neurons, but perhaps due to changes in neural stem cell behavior as well. Sox9 ablation in neural stem cells should generate new neurons and neuroblasts at the lesion site after spinal cord injury and may thus contribute to recovery.
Investigators in Dr. Brown’s laboratory have knocked down *Sox9* specifically in neural stem cells and assessed hind limb functional recovery after a contusion SCI. Although the average BMS score for neural stem cell *Sox9* knockdown mice was 1.30, and only 0.65 for the control mice expressing wild type levels of SOX9, these scores were not significantly different. However, this does not mean that *Sox9* knockdown neural stem cells are not contributing any benefit leading to the functional recovery seen in the *Sox9* knockdown mice. Also, a mouse line expressing Cre recombinase only in astrocytes exists and could be bred to our *Sox9^{flox/flox},Cre* mice. It would be interesting to know if the improvements in motor function described herein using a mouse line in which *Sox9* is knocked down throughout the CNS could be recapitulated by a combination of improvements seen in the neural stem cell *Sox9* knockdowns and astrocyte specific *Sox9* knockdowns.

5.20 Conclusion

*Sox9* knockdown reduces the expression of anti-regenerative extracellular matrix genes, results in increased reactive sprouting and synaptic plasticity, and improves motor function in a mouse model of SCI. The data presented in this thesis demonstrate the potential anti-SOX9 strategies have for treatment of spinal cord injury, and the mechanism leading to anatomic CNS plasticity described herein suggests that anti-SOX9 strategies may in fact be beneficial for the treatment of other types of CNS neurotrauma.
5.21 References


Ethics Approval for Animal Usage on Protocol 2007-009-02

AUP Number: 2007-009-02
AUP Title: Molecular and Cellular Studies of Spinal Cord Injury in Mice and Rats

Yearly Renewal Date: 06/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-009-02 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Mollard, Maureen
on behalf of the Animal Use Subcommittee
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Abstracts:


Presentations:


**Oral:**


McKillop, W.M. SOX9 knockdown promotes reactive sprouting and synaptic plasticity post spinal cord injury. Oral Presentation given to the Molecular Medicince Research Group at the Robarts Research Institute. 2014.


