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Conditional Sox9 ablation 30 days after spinal cord injury: Testing the therapeutic value of a successful acute strategy to increase neuroplasticity in a model of chronic spinal cord injury

Natalie M. Ossowski, *The University of Western Ontario*

Supervisor: Dr. Arthur Brown, *The University of Western Ontario*

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Abstract

Many individuals who have suffered from spinal cord injury (SCI) have longstanding damage. The molecular environment of the spinal cord is not permissive to axonal growth and neuroplasticity after injury is limited. Perineuronal nets containing chondroitin sulfate proteoglycans (CSPGs) are major inhibitors of axonal sprouting. Our laboratory has identified that the transcription factor SOX9 regulates a battery of genes involved in CSPG biosynthesis. Using *Sox9* conditional knockout mice, we have shown that ablating *Sox9* before injury decreases CSPG levels in the cord, increases reparative sprouting, and leads to improved locomotor recovery. However, it is unknown whether *Sox9* ablation following SCI leads to similar recovery. To investigate this, *Sox9* was ablated in mice 30 days after SCI. This ablation did not decrease levels of perineuronal nets, increase neuroplasticity or improve locomotor recovery compared to controls. These results will be discussed in the context of CSPG turnover rate and matrix metalloproteinase activity.

Keywords

Spinal cord injury, Sox9, chondroitin sulfate proteoglycans, perineuronal nets, neuroplasticity, axonal sprouting, matrix metalloproteinases.

Co-authorship Statement

This thesis has been prepared to be published in the future and is co-authored by myself, Russell MacMillan, Dr. Nicole Geremia, Dr. Todd Hryciw, Dr. Kathy Xu, and Dr. Arthur Brown. Russell MacMillan assisted with the quantification of BDA. Dr. Nicole Geremia was the second observer in hindlimb movement assessment using the Basso Mouse Scale. Dr. Todd Hryciw performed the BDA injections to label corticospinal tract fibres and provided guidance when I carried out qPCR and assessed mRNA expression levels. Dr. Kathy Xu assisted with genotyping the mice. I planned and performed the experiments, and carried out the statistical analysis of data with the assistance of those mentioned above. Dr. Andreas Schedl provided the *Sox9*$^{f/f}$ mice. Dr. Arthur Brown provided direction in the design of the study, provided guidance and support, and edited the written thesis.

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List of Abbreviations

AMH – Anti-Müllerian hormone

ANOVA – Analyses of Variance

BDA – Biotinylated dextran amines

BDNF – Brain-derived neurotrophic factor

BMS – Basso Mouse Scale

C4ST – Chondroitin 4-sulfotransferase

cAMP – 3'-5'-cyclic adenosine monophosphate

CD – Campomelic dysplasia

ChABC – Chondroitinase ABC

CNS – Central nervous system

Col4a1 – Collagen alpha-1(IV)

Cre – Cre recombinase

CSPG – Chondroitin sulfate proteoglycan

CST – Corticospinal tract

EGFR – Epidermal growth factor receptor

ER- Estrogen receptor

ERK – Extracellular signal-regulated kinase

GABA – Gamma aminobutyric acid

GAG – Glycosaminoglycan

GAP – Growth associated protein

GFAP – Glial fibrillary acidic protein

JNK – c-Jun N-terminal kinase

KO – Knockout

LAR – Leukocyte common antigen-related phosphatase

LPS – Lipopolysacharide

MMP – Matrix metalloproteinase

NG2 – Neuron-glial antigen 2

OD – Ocular dominance

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

PNN – Perineuronal nets

PNS – Peripheral nervous system

PTPσ – Receptor protein tyrosine phosphatase σ

SCI – Spinal cord injury

SOX – Sry-type HMG box transcription factor

VGLUT1 – Vesicular glutamate transporter 1

WFA – Wisteria Floribunda agglutinin

XT – Xylosyltransferase

Chapter 1: Introduction

1.1 Spinal Cord Injury

The spinal cord is responsible for the transfer of sensory and motor information between the brain and the rest of the body. Damage to the cord interrupts signaling to and from the brain, resulting in debilitating loss of sensation and motor control. In addition to these impairments, individuals with spinal cord injury (SCI) often suffer from chronic pain, spasms, difficulty breathing, sexual dysfunction and loss of bladder and bowel control (Masri and Keller, 2012; Elbasiouny et al., 2010; Zimmer et al., 2007; Hess and Hough, 2012; Benevento and Sipski, 2002). In Canada, it is estimated that 86,000 people live with SCI and about 4,300 new cases occur each year (Noonan et al., 2012; Farry, 2010). Traffic accidents are the most common cause of SCI in North American, followed by falls (from both the ground level and from an elevation) (Singh et al., 2014). Due to the vast impact that SCI has on the health and quality of life of a person, the lifetime cost of an individual living with SCI in Canada can range from $1.5 to $3.0 million (Krueger et al., 2013) and the annual economic burden is $3.6 billion (Farry, 2010). Currently, there is no effective cure for SCI.

The spinal cord is made up of a collection of neurons possessing long extensions called axons, which are responsible for the transport of signals. Injury to the neurons of the cord can result from physical trauma, which includes impact, persistent compression, stretching, and/or laceration (Dumont et al., 2001). This physical trauma is referred to as the primary injury (Dumont et al., 2001). A secondary injury follows the primary and intensifies damage. Ischemia, ischemia reperfusion, excitotoxicity, and immunologic

activation are all secondary injuries that occur following primary injury (Dumont et al., 2001). The physical trauma and secondary damages that occur lead to the destruction of axons, resulting in loss of functional synaptic connections. Axons in the central nervous system do not naturally regenerate after SCI and thus, spontaneous recovery after injury is limited.

1.2 Biochemical Events Following SCI

Immediately following the mechanical trauma of SCI, microglia become activated and transform into macrophages (Popovich et al., 2002). These macrophages secrete inflammatory cytokines, proteases, and reactive oxygen species, which can lead to further cell injury in the cord (Fleming et al., 2006). However, these macrophages also engulf debris and secrete pro-regenerative cytokines and so have beneficial effects as well (Fleming et al., 2006). The blood-spinal cord barrier breaks down from the mechanical trauma of SCI and blood-borne inflammatory cells enter the cord (Hausmann, 2003). The first to arrive at the injury are neutrophils, seen just 4 hours post-SCI, peaking at 1-3 days and remaining high for 10 days in humans (Fleming et al., 2006.) Neutrophils release proteases and reactive oxygen species. Neutrophil elastase breaks down endothelial cells and is thought to increase heamorhage (Hausmann, 2003). In addition to neutrophils, blood-borne macrophages infiltrate the spinal cord through the disrupted blood-spinal cord barrier. Macrophages peak at 5-10 days after injury (Fleming et al., 2006). T-lymphocytes also enter the spinal cord and are thought to kill specific cells and release cytokines (Hausmann, 2003). Lymphocytes seem to only appear in the human spinal cord weeks after injury (Fleming et al., 2006).

In addition to the inflammatory response after SCI, a variety of other biochemical events take place. Resident astrocytes become active, and proliferation and hypertrophy of these cells occur (Hausmann, 2003). Astrocytes secrete extracellular matrix proteins forming a glial scar, and release myelin associated neurite growth inhibitory factors (Hausmann, 2003). This response is thought to inhibit neuroplasticity after SCI. Another event that occurs as a result of SCI is increases of extracellular glutamate to toxic levels (Hausmann, 2003). Too much activation of NMDA glutamate receptors leads to an influx of Ca^{2+} into neurons which, through various secondary processes, results in cell death (Hausmann, 2003). Furthermore, the vascular damage that occurs from SCI results in hypoxia in the cord. A lack of oxygen leads to mitochondrial damage, energy depletion and subsequent cell death (Hausmann, 2003).

At months to a year after SCI, microglia and macrophages are still present at the lesion but macrophages no longer express CD68, a marker of phagocytic macrophages (Fleming et al., 2006). It is unclear what the role of macrophages is in the chronically injured spinal cord (Fleming et al., 2006). In some cases, lymphocytes can be seen weeks to months after SCI (Fleming et al., 2006). In mice with SCI, it was found that B lymphocytes remain active in the spinal cord and systemically at 1 month post-injury, a chronic time-point in the mouse (Ankeny et al., 2006). Furthermore, at 42 days post-injury there were higher levels of autoantibodies to CNS proteins and nuclear antigens in the sera of injured animals than in controls (Ankeny et al., 2006). A subset of these antibodies were found to be neurotoxic and could lead to inflammation (Ankeny et al., 2006). In sera of patients with chronic SCI (greater than 12 months) high amounts of proinflammatory cytokines such as interleukein-2 and tumor necrosis factor alpha, were

found compared to controls (Hayes et al., 2002). These results demonstrate that inflammation after SCI persists well-past the acute phases, and are part of a multitude of studies that claim inflammation persists indefinitely (Schwab et al., 2014).

1.3 Neuroplasticity

Although recovery following SCI is quite limited, a moderate amount of spontaneous recovery does occur (Lynskey et al., 2008). For example, improvements in the ASIA Motor Score can be seen in quadriplegic and paraplegic patients within the first ten weeks following SCI (Curt et al., 2008). In animal models, stepping of rats (assessed using the Basso, Beattie and Bresnahan scale) improves over 42 days following a lateral thoracic hemisection (Ballermann and Fouad, 2006). This spontaneous recovery following SCI has been attributed mainly to the remyelination of spared axons and to neuroplasticity in the cord; that is, axonal sprouting of and the reorganization of spinal circuits (Bareyre et al., 2004; Ballermann and Fouad, 2006; Courtine et al., 2008).

Despite the limited ability of central nervous system (CNS) axons to regenerate, axons in the peripheral nervous system (PNS) are known to readily regenerate following injury (Abe and Cavalli, 2008). After injury, PNS neurons increase the expression of various factors necessary for regeneration such as the mitogen activated protein kinases c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), as well as certain transcription factors induced by cytokine release (Abe and Cavalli, 2008). Additionally, PNS neurons are thought to regenerate more readily because their molecular environment is more permissive to growth. Specifically, Schwann cells surround axons in the PNS, whereas axons in the CNS are surrounded by oligodendrocytes. It is thought that

Schwann cells provide an environment that is more permissive to regeneration, a hypothesis supported by the fact that when PNS and CNS neurons are transplanted into an environment high in Schwann cells, both are able to grow (David and Aguayo, 1981; Harvey and Plant, 1995). However, when transplanted into an environment rich in oligodendrocytes, PNS neurons show lack of growth (Fawcett et al., 1989). The increased production of growth promoting factors by Schwann cells following injury, and the presence of cell adhesion molecules on Schwann cell membranes are thought to contribute to their regeneration supporting properties (Fawcett and Keynes, 1990; Bixby et al., 1988).

1.4 Perineuronal Nets

In contrast to the PNS, the molecular environment of the CNS is not permissive to regeneration. A major barrier to neuroplasticity in the spinal cord is the presence of perineuronal nets (PNN) (Sorg et al., 2016). PNNs are extracellular matrix structures that surround the soma and proximal dendrites of certain neurons (Blosa et al., 2016). Their primary role is to stabilize synaptic connections and inhibit axonal sprouting (Blosa et al., 2016). PNNs form near the end of development, after the establishment of synaptic circuitry and are known to correspond with the end of the critical period (time during development defined by high levels of plasticity in the CNS) (Wang and Fawcett, 2012). In studies where formation of mature synaptic connections during the early postnatal phase was inhibited by blocking normal neuronal activity, the formation of PNNs diminishes (Wang and Fawcett, 2012). For example, dark-rearing cats from birth, which decreases the sensory input of the visual system, prolonged the critical period in their

visual cortex and decreased the expression of Cat-301, Cat-315 and Cat-316 antigen, all components of PNNs (Lander et al., 1997; Wang and Fawcett, 2012).

1.5 Chondroitin Sulfate Proteoglycans

The main inhibitory component of PNNs is chondroitin sulfate proteoglycan (CSPG) (Wang and Fawcett, 2012). CSPGs are made up of a lectican protein core with covalently attached chains of chondroitin sulfate glycosaminoglycans (GAG) (Wang and Fawcett, 2012). The number of GAG sidechains varies for different CSPGs. For example, the CSPG aggrecan contains around 100 GAG sidechains, while brevican and aggrecan contain between 0 to 5 (Siebert et al., 2014). The main group of CSPGs is the lectican group which includes aggrecan, neurocan, brevican and versican in its three isoforms (Siebert et al., 2014). These CSPGs are secreted by astrocytes and neurons. They contain a globular domain (G1) at their N-terminal and a G3 domain at their C-terminal (Siebert et al., 2014). Phosphacan is another type of CSPG and is a variant of the receptor protein tyrosine phosphatase (phosphacan does not have the tyrosine phosphatase intracellular domain (Siebert et al., 2014). The last CSPG is neuron-glial antigen 2 (NG2). NG2 is a transmembrane CSPG found on activated microglia, macrophages, oligodendrocyte progenitor cells and polydendrocytes (Sibert et al., 2014). *In vivo* and *in vitro* studies have demonstrated that axons do not extend into CSPG dense areas (Davies et al., 1997; McKeon et al., 1991). Furthermore, Pizzorusso et al. (2002) demonstrated the inhibitory effect of CSPGs on neuroplasticity in a study investigating plasticity in ocular dominance columns. During the postnatal phase, blocking sensory input from one eye (monocular deprivation) causes a shift in ocular dominance (OD) to the non-deprived eye (Pizzorusso

et al., 2002). However, in adulthood this ocular dominance shift does not occur. CSPGs in the brain are thought to inhibit axonal sprouting and consequently the ocular dominance shift (Pizzorusso et al., 2002). The authors injected chondroitinase ABC (ChABC), a bacterial enzyme that degrades CSPGs, into the visual cortex of rats and OD plasticity was evaluated. OD shifted towards the non-deprived eye in ChABC treated rats, demonstrating that CSPGs present in the visual cortex block activity dependent learning and neuroplasticity from occurring in the adult and that their degradation could create a new window of neuroplasticity (Pizzorusso et al., 2002).

Within days following SCI, levels of CSPGs increase in the lesion area of the cord forming a major component of the glial scar (Lemons et al., 1999). A significant source of this increase in CSPGs is thought to be astrocytes (Lemons et al., 1999). Specifically, levels of the CSPGs neurocan, NG2, versican and brevican increase (Jones et al., 2003). Neurocan and versican levels remain elevated for 4 weeks after injury, while brevican levels are elevated for 2 months (Jones et al., 2003). NG2 peaks at 1 week following injury and remains increased for at least 7 weeks post-injury (Jones et al., 2002). The rise in CSPGs at the lesion is believed to protect intact tissue around the lesion from degradative enzymes present after SCI and from the inflammatory cells that lead to secondary damages (Fitch and Silver, 1997). However, the injury induced rise in CSPGs can create a microenvironment that inhibits neuroplasticity.

As discussed above, CSPGs are inhibitory to axonal sprouting and are known to be an impediment to SCI recovery (Jones et al., 2003; Lemons et al., 1999; Wang and Fawcett, 2012). CSPGs are thought to act as both a physical and molecular barrier to axonal growth. They bind to cell and substrate adhesion molecules, which are necessary for cell

migration, and block axons from binding these molecules (McKeon et al., 1995). CSPGs can also bind to growth factors such as heparin binding growth factor, and repulsive guidance molecules such as semaphorin 3A to modulate neuroplasticity (Deepa et al., 2002; De Wit et al., 2005; Williams et al., 2010). In addition to acting as a binding scaffold, CSPGs have recently been shown to act as ligands to receptor protein tyrosine phosphatase σ (PTPσ) and leukocyte common antigen-related phosphatase (LAR) (Shen et al., 2009; Fry et al., 2010; Fisher et al., 2011). CSPG-mediated activation of PTPσ is thought to contribute to axonal growth inhibition since disruption of the PTPσ gene allows axons to infiltrate regions high in CSPGs (Shen et al., 2009). Blockade of LAR also eliminates CSPG mediated neurite growth inhibition and leads to locomotor recovery in mice after SCI (Fisher et al., 2011). Lastly, CSPGs are known to activate epidermal growth factor receptor (EGFR) leading to autophosphorylation of the receptor (Koprivica et al., 2005). When the kinase activity of EGFR was blocked, CSPG-mediated neurite outgrowth inhibition was neutralized, demonstrating that activation of EGFR may also be a mechanism through which CSPGs act to limit neuroplasticity (Koprivica et al., 2005).

1.6 Targeting CSPGs as treatment of SCI

Anti-CSPG treatments for SCI show promise in increasing neuroplasticity and improving recovery following injury. Bradbury et al. (2002) were the first to demonstrate that treatment of rats with ChABC leads to functional improvement following SCI. The authors administered ChABC intrathecally in rats immediately after a C4 dorsal column crush (Bradbury et al., 2002). They found that there was increased regeneration of injured dorsal column axons and of corticospinal tract (CST) axons compared to control rats

(Bradbury et al., 2002). Furthermore, electrical stimulation of the motor cortex resulted in large postsynaptic cord dorsum potentials compared to controls, showing that CST functional connections increased (Bradbury et al., 2002). Lastly, ChABC treatement improved locomotor recovery as assessed by beam and grid walk (Bradbury et al., 2002).

Another method of reducing CSPGs is by targeting their synthesis. Grimpe and Silver (2004) decreased the expression of xylosyltransferase-1 (XT-1), an enzyme that initiates glycosylation of the protein backbone in CSPGs. By blocking glycosylation, the amount of GAG chains was reduced in the lesion and there was increased axonal regeneration of transplanted dorsal root ganglia axons around the lesion (Grimpe and Silver, 2004). In another study carried out by Takeuchi et al. (2015) the gene encoding the enzyme *N*-acetylgalactosaminyltransferase-1 was knocked out. This enzyme is responsible for the synthesis of the CSPG polysaccharide chain and thus knockout (KO) mice showed lower CSPG levels (Takeuchi et al., 2015). The KO animals also had higher serotonin immunoreactivity and increased corticospinal tract labeling, demonstrating that axon regrowth and/or sprouting occurred. Finally, the KO animals showed improved locomotor recovery as assessed with the Basso Mouse Scale (BMS) (Takeuchi et al., 2015).

1.7 Identification of the transcription factor SOX9 as a regulator of CSPG levels

Despite the ability of ChABC to improve recovery after SCI in animals, the enzyme has characteristics that make it challenging to apply in humans. ChABC is not thermally stable, has a short half-life, and is not able to effectively cross the blood-brain-barrier (Nazari-Robati et al., 2013; Zhao et al., 2011; Yang et al., 2015). Furthermore, a host of enzymes contribute to the synthesis of CSPGs thus, an upstream target would be a

more ideal candidate for treatment of SCI. XT-1 activity as well as activity of its isoform XT-2, are needed for the initiation of side chain synthesis (Grimpe and Silver, 2004; Ponighaus et al., 2007). These side chains are then sulfated, a process carried out by chondroitin 4-sulfotransferase (C4ST) (Yamauchi et al., 2000). In 2007, our lab sought to identify key regulators of XT-1, XT-2 and C4ST gene expression (Gris et al., 2007). Using *in silico* analysis, common transcription factor binding sites on the promoter regions of the three enzymes were identified in mouse, rat and human genes. SOX9 was identified as one of several transcription factors that could possibly regulate expression of XT-1, XT-2 and C4ST (Gris et al., 2007). Furthermore, when the expression profile of SOX9 was analyzed, it was found that 12 hours after SCI, SOX9 expression increased 12-fold (Gris et al., 2007). This suggests that SOX9 could be an important player in CSPG formation post-SCI.

1.8 SOX9

SOX9 is a member of the HMG-box class DNA binding proteins. This group of proteins recognizes a specific sequence in the minor groove of DNA (Lefebvre et al., 1997). Three of the most well-known functions of the SOX9 transcription factor are male sex development, gliogenesis and chondrocyte differentiation. SOX9 interacts with steroidogenic factor 1 in Sertoli cells to increase expression of anti-Müllerian hormone (AMH) (Tanaka and Nishinakamura, 2014). AMH is needed for regression of the Müllerian duct, which is a critical step in developing the male phenotype. A mutation in SOX9 leads to autosomal sex reversal (Kwok et al., 1995).

In the CNS, glial cells express SOX transcription factors, and it was found that SOX9 specifically is involved in gliogenesis (Stolt et al., 2003). Mice with *Sox9* ablated in neural stem cells have decreased levels of oligodendrocyte progenitors and astrocytes, but increased levels of motoneurons (Stolt et al., 2003). Furthermore, it was found that blocking SOX9 function in the subventricular zone using microRNA increases neuron cell number (Cheng et al., 2009).

In addition to autosomal sex reversal and disrupted gliogenesis, mutations in SOX9 often lead to the skeletal malformation syndrome Campomelic dysplasia (CD) (Kwok et al., 1995). Some of the cardinal features of CD include bowing and angulation of the femora and tibiae, hypoplastic scapulae, 11 pairs of ribs, dislocated hips, and pelvic abnormalities (Schafer et al., 1996). Non-skeletal abnormalities also occur including lack of olfactory bulb and tract formation, and cardiac and renal defects (Schafer et al., 1996). Most individuals afflicted with this congenital disease die within 1 week from cardiac failure (Schafer et al., 1996). A condition known as Pierre Robin Sequence can also arise when there are alterations in SOX9 activity due to mutations in chromosomal regions near the SOX9 gene (Selvi and Mukunda Priyanka, 2013). Pierre Robin Sequence results in abnormal development of facial features often leading to a small mandible, cleft palate, and glossoptosis (Selvi and Mukunda Priyanka, 2013).

Dysregulation of SOX9 is thought to lead to the facial malformations in Pierre Robin Sequence and some of the skeletal abnormalities in Campomelic dysplasia due to its major role in regulating chondrogenesis (Selvi and Mukunda Priyanka, 2013; Ikeda et al., 2005). Bi et al. (1999) established $Sox9^{-/-}$ embryonic stem cells and examined the fates of $Sox9^{-/-}$ cells in mouse embryo chimeras. They found that in cartilage, $Sox9^{-/-}$ cells did

not undergo chondrogenic mesenchymal condensation; that is, they were arrested as mesenchymal cells and did not express chondrocyte markers Col2a1, Col9a2, Col11a2, and aggrecan (Bi et al., 1999). Furthermore, in teratomas that were derived from the *Sox9*$^{-/-}$ embryonic stem cells, no cartilage formed (Bi et al., 1999). In another study, *Sox9* expression was ablated in limb buds before chondrogenic mesenchymal condensation (Akiyama et al., 2002). The ablation of *Sox9* stopped chondrogenic mesenchymal condensation from occurring and resulted in the absence of cartilage and bone (Akiyama et al., 2002). This data demonstrates that SOX9 is necessary for the first step of chondrogenesis; the condensation of mesenchymal stem cells. The transcription factor also plays a role in regulating later stages of chondrogenesis. SOX9 binding sites are present in the enhancer regions of the chondrocyte related genes Col2a1, Col11a2 and CD-RAP (Lefebvre et al., 1997; Bridgewater et al., 1998; Xie et al., 1999). SOX9 is known to activate these enhancer regions and increase aggrecan expression *in vitro,* and to directly promote expression of Col2a1 *in vivo* in mice (Lefebvre et al., 1997; Bridgewater et al., 1998; Xie et al., 1999; Sekiya et al., 2000; Bell et al., 1997). In embryos where *Sox9* expression was ablated after mesenchymal condensation, severe chondrodysplasia was present. The majority of cells remained as condensed mesenchymal cells (Akiyama et al., 2002). Because SOX9 plays a major role in the synthesis of extracellular matrix components, it was the ideal candidate to investigate as a key regulator in the formation of the SCI glial scar.

1.9 SOX9 and CSPG related gene expression

The first step in assessing whether SOX9 is a key player in the formation of the CSPG rich glial scar was to test whether it regulates the expression of CSPG-related genes. Gris et al. (2007) transfected rat primary astrocytes with a CMV-SOX9 expression construct to upregulate expression of the transcription factor. Increased *Sox9* expression resulted in increased mRNA levels of XTI, XTII and C4ST (Gris et al., 2007). Furthermore, when *Sox9* expression was knocked down using anti-SOX9 siRNA, levels of XTI, XTII and C4ST were significantly reduced (Gris et al., 2007). Similar expression patterns were discovered *in vivo* when *Sox9* expression was ablated in the injured mouse spinal cord (McKillop et al., 2013). In this study, in addition to XTI and C4ST mRNA levels being reduced, other CSPG and glial scar-related genes including aggrecan, brevican, neurocan, Col2a1 and Col4a1 were reduced two weeks post-SCI (McKillop et al., 2013). Levels of CSPG protein and perineuronal net matrix were also reduced in *Sox9* KOs compared to controls (McKillop et al., 2013; McKillop et al., 2016). These results demonstrate that SOX9 regulates levels of CSPGs and could be targeted as a way of increasing neuroplasticity and improving recovery after SCI.

1.10 Ablation of *Sox9* prior to SCI increases reparative sprouting and improves locomotor recovery

The next step in our laboratory's investigation was to determine whether ablation of *Sox9* could increase neuroplasticity and improve locomotor recovery after SCI. To assess this, *Sox9* expression was ablated prior to a T9 SCI. Neuroplasticity in the *Sox9* KO and control mice was assessed by the levels of serotonin, synaptophysin, and vesicular glutamate transporter 1 (VGLUT1) immunoreactivity below the level of the

lesion, and by anterograde tract tracing studies. Hindlimb locomotor recovery in *Sox9* KOs and controls was assessed using the BMS. Ten weeks post-SCI, levels of serotonin immunoreactivity were found to be higher in *Sox9* KO mice than in controls, immediately caudal to the lesion and in the lumbar enlargement (McKillop et al. 2016). In the anterograde tract tracing studies, biotinylated dextran amines (BDA) was injected into the motor cortex of control and *Sox9* KO mice. Two weeks later, BDA levels around neurons below the level of the lesion were quantified. The amount of BDA positive puncta surrounding neurons in the ventral-half of the cord was increased in *Sox9* KO mice compared to controls (McKillop et al., 2016). These results demonstrate that there is increased neuroplasticity in the cord following injury when *Sox9* is ablated. To determine whether *Sox9* KOs have increased neural input caudal to the lesion, synaptophysin and VGLUT1 were assessed. Increases were found in the number of synaptophysin+ and VGLUT1+ puncta, as well as the area of immunoreactivity for both markers in *Sox9* KO mice compared to controls (McKillop et al., 2016). Furthermore, VGLUT1 was shown to co-localize with BDA demonstrating increased VGLUT+ release sites on the BDA labeled fibres (McKillop et al., 2016). Lastly, *Sox9* KO mice had a higher BMS score at 14 weeks post-SCI demonstrating that they recovered their hindlimb function more than controls (McKillop et al., 2013). Thus, conditional *Sox9* ablation prior to SCI leads to reduced CSPG levels in the injured cord and improved locomotor recovery by increasing reparative sprouting. However, given that the majority of people with SCI have long-standing disease, will conditional *Sox9* ablation weeks after SCI also lead to reductions in CSPG levels, increased reparative sprouting and improved locomotor recovery? Investigating this question is the aim of the current study.

1.11 Rationale for current study

It is important to determine whether delayed ablation of *Sox9* can increase neuroplasticity and improve recovery because many people have longstanding spinal cord injuries. These individuals would require treatment months to years after their injury, and my study will investigate whether targeting SOX9 in chronic SCI will be effective. In addition, targeting SOX9 weeks after SCI can help to define a window of treatment opportunity.

There is contrasting support in the literature regarding whether ablating *Sox9* weeks after SCI will increase axonal sprouting and improve recovery. When Wang et al. (2011) administered ChABC to degrade PNNs in spinal cord injured rats 1 month after injury, they found increased numbers of axonal branches from corticospinal tract fibres crossing into both the grey and white matter rostral to the lesion. Furthermore, ChABC-treated animals had increased colocalization of VGLUT1- and BDA-positive puncta in the dorsal CST, demonstrating that the CST fiber sprouting was associated with an increased presence of synaptic elements (Wang et al., 2011). However, rats did not show improvements in skilled paw reaching, ladder walk, or beam walk compared to penicillinase-treated controls. Another group, Lee et al. (2012), administered ChABC to mice three weeks post-SCI and observed improvements in hindlimb movement, assessed using the BMS. When the authors investigated levels of neuroplasticity by measuring densities of choline acetyletransferase-expressing cholinergic terminals and vesicular gamma aminobutyric acid (GABA) transporter-positive synaptic terminals around motorneurons in the ventral horn, they found no differences in ChABC-treated mice

versus controls (Lee et al., 2012). Furthermore, VGLUT1-positive synaptic terminals were quantified in lamina VII and IX and again, no differences were found between ChABC-treated and control mice (Lee et al., 2012). These results suggest that ChABC was not able to increase synaptic plasticity of cholinergic and inhibitory synapses in treated injured mice. Lastly, in a study carried out by Carter et al. (2011), spinal cord injured mice were treated with ChABC one month after injury. It was found that chondroitinase treatment had a neuroprotective effect on rubrospinal tract neurons in these animals. Delayed treatment was able to rescue soma atrophy and restore cell size eight weeks post-SCI (Carter et al., 2011).

Some of the results from these studies demonstrate the possibility of increased neuroplasticity and locomotor recovery when CSPGs are degraded weeks after SCI. These results also support my rationale that ablating *Sox9* weeks after SCI can have similar effects.

1.12 Hypothesis and Prediction

I hypothesize that CSPG-rich PNNs limit neuroplasticity and recovery following SCI. I predict that ablating *Sox9* several weeks after SCI will decrease PNN levels, increase neuroplasticity, and improve locomotor recovery in mice.

Chapter 2: Materials and Methods

2.1 *Sox9* Conditional KO Mice

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* conditional KO mice were used to investigate the effect of *Sox9* ablation after SCI. A mouse strain (from Dr. Andreas Schedl) with floxed *Sox9* alleles (loxP sites surrounding exon 2 and 3 of *Sox9*) (Akiyama et al., 2002) was bred with a transgenic mouse line expressing the enzyme Cre recombinase (Cre) fused to a mutated ligand binding domain of the mouse estrogen receptor (ER) under the control of a chimeric promoter/enhancer of the cytomegalovirus immediate-early enhancer and the chicken beta globin promoter/enhancer (Hayashi and McMahon, 2002). (Jackson Laboratories, Bar Harbor, Maine). Two groups of offspring were generated from this breeding; $Sox9^{flox/flox}$*;CAGGCreER* mice (referred to as $Sox9^{flox/flox}$*;Cre*) which were used as the conditional KO experimental group and $Sox9^{flox/flox}$ mice which were used as the control group. The Cre recombinase fused estrogen receptor (Cre-ER) does not bind estrogen, but readily binds the drug tamoxifen (Hayashi and McMahon, 2002). Tamoxifen administration was used to ablate *Sox9* in the $Sox9^{flox/flox}$*;Cre mice*, as when the drug binds Cre-ER in the cytoplasm, the complex is then free to enter the nucleus where it excises exons 2 and 3 of *Sox9* (Hayashi and McMahon, 2002). Mice were genotyped using ear notch DNA amplified by polymerase chain reaction (PCR) (40 cycles) using the following primers:

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

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

Sox9$^{knock\text{-}out}$ allele: 5’-GTCAAGCGACCCATG-3’ and 5’-TGGTAATGAGTCATACACAGTAC-3’.

Cre^{+} allele: 5’-CAATTTACTGACCGTACAC-3’ and 5’-AGCTGGCCCAAATGTTGCTG-3’.

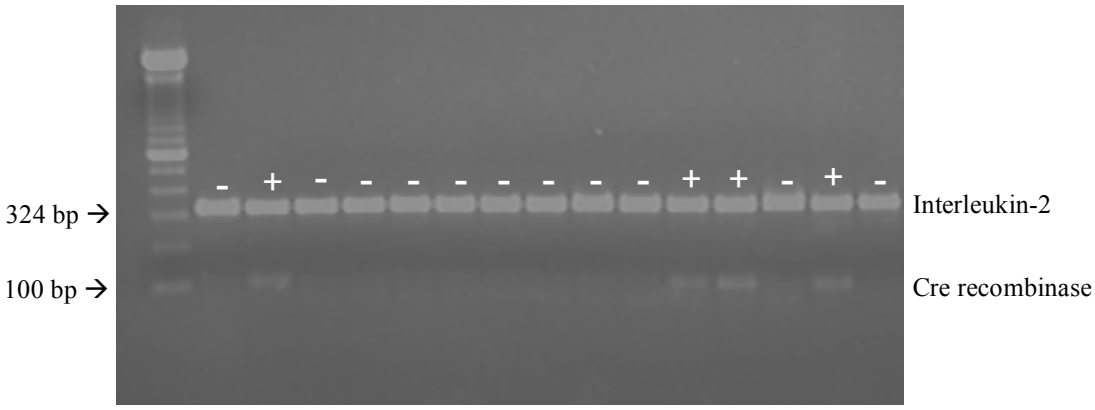


Figure 1. Representative gel of PCR products for the genotyping of Cre recombinase. PCR products were run on 2% agarose gel and a 100 bp ladder was used. Top row of bands shows loading control interleukin-2 (324 bp) and bottom row of bands shows the presence or absence of Cre recombinase (100 bp). Plus symbol signifies presence of Cre gene and minus symbol signifies its absence.

Three weeks following SCI both experimental and control mice were treated with Tamoxifen (Sigma Aldrich, St. Louis, Missouri) dissolved in corn oil at 3mg/20g by oral gavage once a day for 7 days.

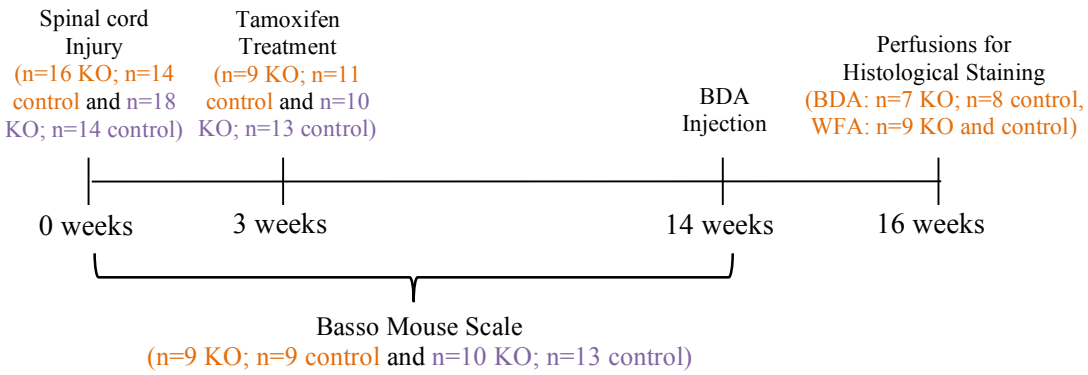
2.2 Spinal Cord Injury

Three weeks prior to tamoxifen treatment, 43 *Sox9*$^{flox/flox}$*;Cre* and 42 *Sox9*$^{flox/flox}$ female mice weighing 20-25 grams were anesthetized using 3% isoflurane and maintained at 1.5% isoflurane and 30% oxygen. A dorsal laminectomy was performed to expose the L1 spinal cord segment. Forceps were used to stabilize the spinal cord at T12 and L2. A 70 kdyne contusion SCI with a 1 second dwell time and 500-1000 μm displacement was performed at L1 using the computer controlled Infinite Horizons Impactor (Precision Systems and Instrumentation, Fairfax, Virginia). SCI surgeries were also performed at the T9 level on 18 *Sox9*$^{flox/flox}$*;Cre* and 14 *Sox9*$^{flox/flox}$ mice. Mice were given buprenorphine (0.01 mg/kg, Schering-Plough, Hertfordshire, UK), Baytril (20 mg/kg, Bayer, Toronto, Ontario, Canada) and 1 mL of 0.9% saline solution subcutaneously immediately after surgery. Buprenorphine and Baytril injections were continued for three days post SCI. Mice were housed individually for a week following surgery to ensure sutures were not pulled out by fellow mice during the healing process, and then were pair or triple housed. Bladders of mice were manually expressed twice daily throughout the experiment.

2.3 *Sox9*, Neurocan, Aggrecan, GFAP and Col4a1 Expression Following Tamoxifen Administration

To determine how long after tamoxifen administration *Sox9* mRNA levels are significantly reduced, a subset of $Sox9^{flox/flox}$ (control) and $Sox9^{flox/flox}$*;Cre* (KO) mice with an L1 injury was perfused at 0, 3, 6, 9 and 14 days after the first tamoxifen administration (Figure 2). These animals were also used to study the expression levels of the CSPGs neurocan and aggrecan, as well as glial fibrillary acidic protein (GFAP) a marker of astrocyte activation, and collagen alpha-1(IV) (Col4a1) a glial scar gene. For RNA quantification, mice were cardiac perfused with 0.9% saline, and 5 mm segments of spinal cord were removed from the lesion and lumbar area below the lesion. Quantitative PCR was used to quantify RNA levels.

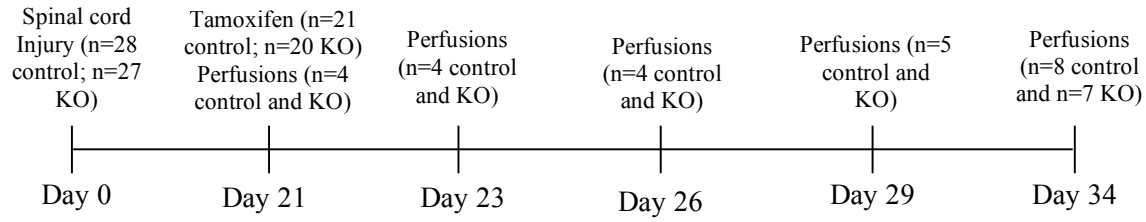
A **SCI recovery following delayed *Sox9* ablation experiment timeline**



Reductions in animal group number
Tamoxifen treatment- 2 KO and 1 control due to too high BMS score at 24hr, 4 KO and 1 control showed self-mutilation, 1 KO and 1 control had poor recovery/were ill
BMS and Wisteria Floribunda Agglutinin (WFA)- 1 control was an outlier and 1 control died
BDA- 2 KO and 1 control due to insufficient labeling efficiency
Tamoxifen treatment- 1 control and 5 KO due to too high BMS score after 24hr, 1 KO showed self-mutilation, and 2 KO from spasm due to bladder expression

* Orange represents animals in L1 lesion study and purple represents T9 lesion study

B ***Sox9*, Neurocan, Aggrecan, GFAP and Col4a1 expression after tamoxifen administration experiment timeline**



Reductions in animal group numbers:
2 KO and 1 control due to self-mutilation
1 KO and 1 control due to poor recovery/were ill
1 control from spasm due to bladder expression

Figure 2. Experimental timelines. A) Timeline of experiment assessing SCI recovery in *Sox9* KO mice versus controls following *Sox9* ablation weeks after injury. B) Timeline of experiment quantifying *Sox9*, neurocan, aggrecan, GFAP and Col4a1 mRNA levels at various time-points following the administration of tamoxifen.

2.4 Quantitative PCR

Spinal cord tissue was placed in 1 mL of TRIzol (Life Technologies, Carlsbad, California) and lysed on ice using a syringe and 25-gauge needle. The tissue was stored in TRIzol at -80°C. Chloroform was mixed into the tissue containing solution and centrifuged at 4°C. The aqueous layer was removed and saved. Equal parts of 70% ethanol were mixed into the aqueous solution. RNA was purified using the RNeasy Mini Kit (QIAGEN, Valencia, California). Complementary DNA was synthesized using the high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, California) and the GeneAmp® PCR System 2400 device (Perkin Elmer, Waltham, Massachusetts). The following thermal cycler conditions were used; 10 min at 25°C, 2 hours at 37°C, and held at 4°C. Taqman® gene expression assays were performed using the primer probe sets listed in Table 1 (Applied Biosystems, Foster City, California).

Table 1. Taqman® Real Time PCR Primer Probe Sets

Primer Probe	Catalog Number	PCR CT Range
GAPDH	Mm99999915_g1	17.405-21.239
Sox9	Mm00448840_m1	23.143-28.237
GFAP	Mm01253033_m1	17.312-21.036
Neurocan	Mm00484007_m1	24.391-27.964
Collagen 4A1	Mm01210125_m1	24.307-28.145
Aggrecan	Mm00545807_m1	25.031-30.728

A ViiA 7 Real-Time PCR System was used for the gene expression assays. The following thermal cycler conditions were used; 95°C for 20 seconds followed by 40 cycles at 95°C for 1 second, and 60°C for 20 seconds. Data was collected at the end of every cycle.

2.5 Corticospinal Tract Anterograde Labeling

BDA (10 000 MW, D1956, Invitrogen, Waltham, MA) injected into the hindlimb motor cortex was used to label corticospinal tract fibres to assess levels of neuroplasticity in the spinal cord. At 14 weeks post-SCI mice were anesthetized and stabilized using a stereotaxic frame. A burr hole, 1.5 mm in diameter, was drilled over the right primary motor cortex. A Hamilton syringe (33 gauge needle) containing 0.4 μL 10% BDA in phosphate-buffered saline (PBS) (10,000 d, Molecular Probes, Invitrogen) was placed into the burr hole and lowered into the brain parenchyma at a depth of 0.5 mm from the cortical surface in four different areas surrounding the location of +1.5 mm lateral, -1 mm posterior to bregma (+1 mm lateral, -0.5 mm posterior, +2 mm lateral, -0.5 mm posterior, +1 mm lateral, -1.5 mm posterior; +2 mm lateral, -1.5 mm posterior) (Pronichev and Lenkov, 1998). BDA (0.1 μL per injection) was then injected into the cortex and the needle maintained in position for 1 min not to disrupt BDA diffusion. Two weeks after BDA injections, mice were cardiac perfused.

2.6 Spinal Cord Sectioning

At 16 weeks post-SCI animals were deeply anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg) and cardiac perfusion was performed. Approximately 50

mL of 0.9% saline was first used to clear the animal's vascular bed. This was followed by 100 mL of 4% paraformaldehyde (4% PFA in 0.1 M phosphate buffer at pH 7.4). Spinal cords were removed and post fixed in 4% PFA for 4 hours. Cords were then transferred to and stored in 10% sucrose solution in 0.1 M phosphate buffer at pH 7.4, overnight at 4°C followed by 24 hours in 20% sucrose solution for cryoprotection. Tissue-Tek Optimum Cutting Temperature Compound (Sakura Finetek U.S.A. Inc, Torrance, California) was used over dry ice to embed and freeze spinal cords. Embedded cords were stored at -80°C until sectioned. Spinal cords were sliced into 16 µm thick cross-sections using a cryostat and thaw mounted onto Superfrost™ Plus glass microscope slides (Fisher Scientific Company, Ottawa, Canada). Slides with spinal cord sections were stored at -20 °C.

2.7 Histological Staining

Slides were thawed at room temperature for 30 minutes before being washed twice for ten minutes in 1% PBS solution. Sections were then treated with 5% goat serum and 0.5% triton-x-100 in PBS, and incubated in a humidified chamber at room temperature for two hours. For perineuronal net staining, slides were treated with biotinylated Wisteria Floribunda agglutinin (WFA, 1:100, Sigma Aldrich, St. Louis, Missouri) and incubated overnight at 4°C in the humidified chamber. The next day, slides were washed again three times for 10 minutes in 1% PBS solution and treated with streptavidin conjugated Alexa-Fluor 488 (1:750, Invitrogen, Carlsbad, California) and incubated at room temperature for two hours. For BDA staining, sections were treated with streptavidin conjugated Alexa-Fluor 488 (1:1000, Invitrogen, Carlsbad, California) for 45 minutes at room temperature. Slides were then washed in 1% PBS three times for

15 min, rinsed briefly in distilled water, and mounted with coverglass using VECTASHIELD® mounting media with DAPI (Vector Laboratories, Burlingame, California).

2.8 Quantification of WFA and BDA

Histological staining was quantified using ImageJ Pro Plus (Media Cybernetics Inc, Bethesda, Maryland). Ten 16 µL thick spinal cord sections spaced 160 µL apart at the lumbar enlargement below the L1 lesion were analyzed for each animal. For WFA quantification, the right and left ventral horns were assessed in each spinal section whereas for BDA, both ventral and dorsal horns were assessed. WFA was quantified by setting a threshold for pixel intensity for positive staining in each image of the right and left ventral horn and the area of positive staining was used for analyses. BDA was quantified by counting the number of BDA positive fibres with a minimum length of 5 µm. The amount of BDA positive staining at T10 was used to assess BDA labeling efficiency.

2.9 Behavioral Testing

The BMS open field locomotor score was used to assess locomotor recovery in the mice (Basso et al., 2006). Two viewers, blind to the genotypes of the animals, evaluated each mouse 24 hours following SCI and mice with BMS scores greater than 0.5 were excluded from the study so that the level of paralyses between all animals was equivalent. Locomotor activity was scored using the BMS weekly for 14 weeks post injury (Figure 2).

2.10 Statistical Analyses

Mean values are expressed as ± SE. WFA and BDA histological staining was subjected to statistical analysis using the Student's T-test. *Sox9* and SOX9 target gene mRNA levels were subjected to two-way analyses of variance (ANOVA) with Šídák multiple comparisons test. BMS scores were subjected to two-way repeated measures ANOVA. All statistical tests were run using GraphPad Prism 6 software (GraphPad Software Inc, La Jolla, California) and a value of $p < 0.05$ was considered significant.

Chapter 3: Results

3.1 *Sox9* mRNA expression levels after onset of tamoxifen administration

To assess the effects of ablating *Sox9* weeks after SCI, a tamoxifen inducible conditional *Sox9* knockout model was used. Tamoxifen was administered to $Sox9^{flox/flox};Cre$ and $Sox9^{flox/flox}$ control mice three weeks post-SCI, once a day, for one week. It was unknown at what time-point following the onset of tamoxifen treatment, levels of *Sox9* mRNA would be significantly reduced. Quantitative PCR was run to assess levels of *Sox9* in the injured spinal cord at 0, 3, 6, 9 and 14 days following the start of tamoxifen treatment at the lesion and in the lumbar cord caudal to the lesion (here after referred to as 'lumbar'). Nine days after the onset of tamoxifen administration, *Sox9* mRNA levels were significantly reduced in the $Sox9^{flox/flox};Cre$ mice compared to controls at the lesion and lumbar spinal cord (Fig. 3). Thus, *Sox9* was ablated by 30 days post-SCI. By day 14 after the onset of tamoxifen, *Sox9* expression was reduced by approximately 74.4% at the lesion (Fig. 3A) and 85.3% in the lumbar (Fig. 3B) spinal cord.

3.2 *Neurocan, Aggrecan, Gfap* and *Col4a1* mRNA expression levels after onset of tamoxifen administration

Messenger RNA levels of genes known to be regulated by SOX9, which are implicated in glial scar formation and astrocyte activation, were also assessed (Gris et al., 2007). Neurocan levels were reduced at 6 and 14 days post-tamoxifen administration at both the lesion and lumbar spinal cord (Fig. 4A), while there was no difference in aggrecan expression (Fig. 4B). GFAP was significantly reduced 14 days after tamoxifen

administration at the lesion and lumbar area (Fig. 4C). No differences were found in the levels of Col4a1 (Fig. 4D).

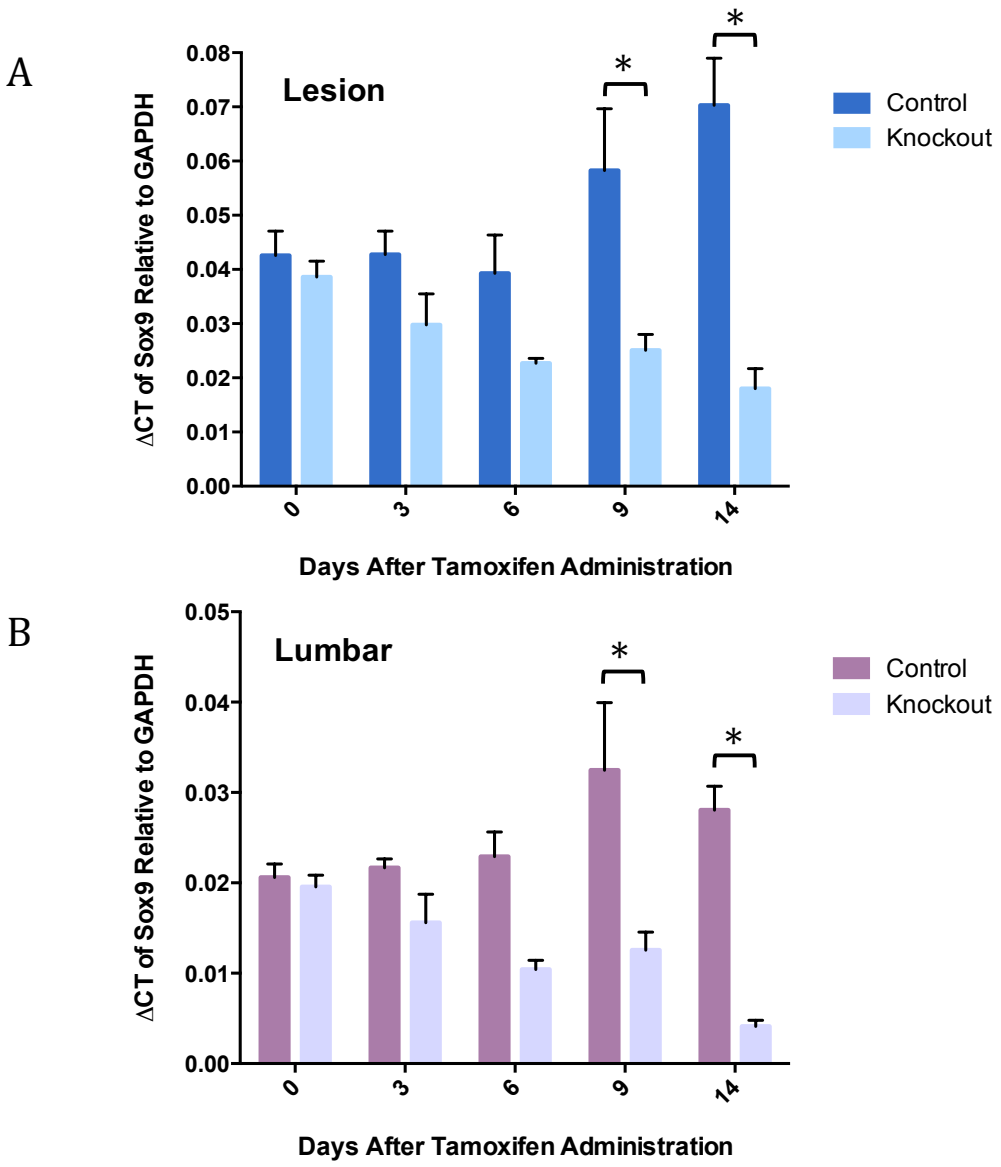
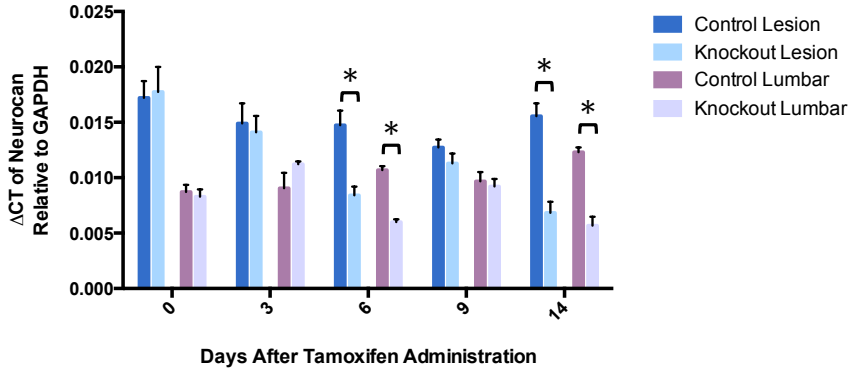
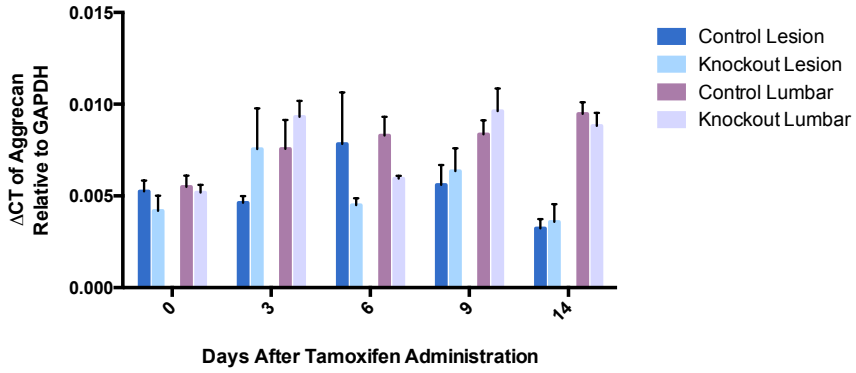


Figure 3. *Sox9* mRNA levels at various time-points following the onset of tamoxifen administration. *Sox9* levels are significantly reduced in *Sox9*$^{f/f}$*;Cre* mice after 9 days following tamoxifen treatment at the lesion (A) and lumbar (B) spinal cord. By day 14, *Sox9* expression is reduced by approximately 74.4% at the lesion (A) and 85.3% in the lumbar (B) spinal cord. (* indicates statistically significant difference between groups, $p < 0.05$, Two-way ANOVA, Šídák multiple comparisons test; n=5 control and n=5 KO for day 9, n=8 control and n=7 KO for day 14).

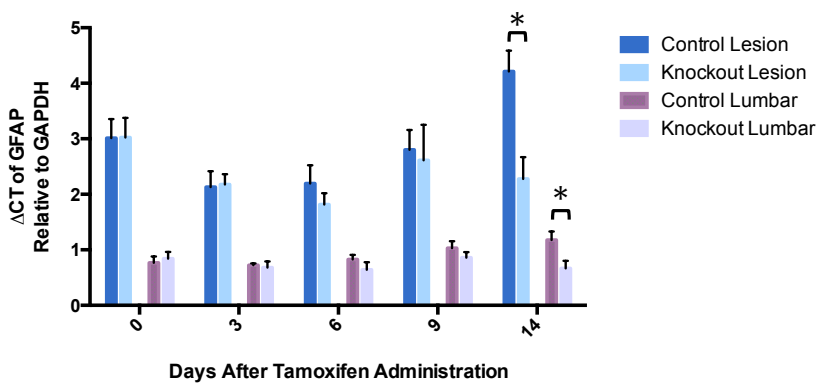
A


ΔCT of Neurocan
Relative to GAPDH
0.025
0.020
0.015
0.010
0.005
0.000
0
3
6
9
14
Days After Tamoxifen Administration
Control Lesion
Knockout Lesion
Control Lumbar
Knockout Lumbar
*
*
*

B


ΔCT of Aggrecan
Relative to GAPDH
0.015
0.010
0.005
0.000
0
3
6
9
14
Days After Tamoxifen Administration
Control Lesion
Knockout Lesion
Control Lumbar
Knockout Lumbar

C


ΔCT of GFAP
Relative to GAPDH
5
4
3
2
1
0
0
3
6
9
14
Days After Tamoxifen Administration
Control Lesion
Knockout Lesion
Control Lumbar
Knockout Lumbar
*
*

D

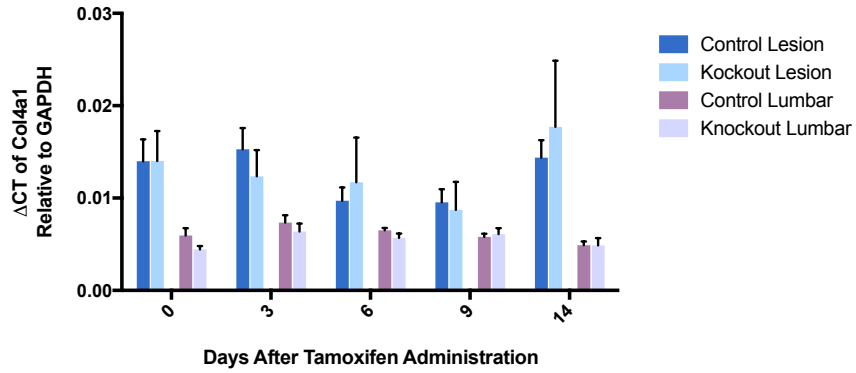

ΔCT of Col4a1
Relative to GAPDH
0.03
0.02
0.01
0.00
0
3
6
9
14
Days After Tamoxifen Administration
Control Lesion
Kockout Lesion
Control Lumbar
Knockout Lumbar

Figure 4. ***Neurocan, aggrecan, GFAP*** **and** ***Col4a1*** **mRNA expression levels following the onset of tamoxifen administration.** Neurocan expression is significantly reduced at Day 6 and 14 after tamoxifen treatment in *Sox9$^{f/f}$;Cre* mice compared to controls (A) while there are no differences in aggrecan mRNA levels (B). GFAP expression is significantly reduced at Day 14 following tamoxifen treatment (C). No differences in Col4a1 expression were found between *Sox9$^{f/f}$;Cre* mice and controls after tamoxifen treatment (D). (* indicates statistically significant difference between groups, $p < 0.05$, Two-way ANOVA, Šídák multiple comparisons test; n=4 control and KO Day 6, n= 8 control and n=7 KO Day 14).

3.3 *Sox9* conditional knockout mice do not have reduced levels of perineuronal net matrix

Perineuronal nets containing CSPGs limit neuroplasticity and recovery after SCI (Sorg et al., 2016). Our laboratory has shown that SOX9 regulates a battery of genes necessary for CSPG production (Gris et al., 2007; McKillop et al., 2013). When *Sox9* expression is ablated at the time of SCI, levels of CSPGs and PNNs decrease (McKillop et al., 2013; McKillop et al., 2016). However, it is unknown whether ablating *Sox9* weeks after SCI also reduces levels of PNNs. To address this question, mice that had *Sox9* expression ablated 30 days post-SCI were used to assess levels of PNNs in the lumbar cord caudal to an L1 lesion. Spinal cord cross-sections were stained with WFA, a lectin that visualizes PNNs, and the area of positive staining in the ventral horns caudal to the lesion was assessed. No differences in the levels of PNNs were found between $Sox9^{f/f};Cre$ mice (*Sox9* KO) and $Sox9^{f/f}$ control mice (Figure 5).

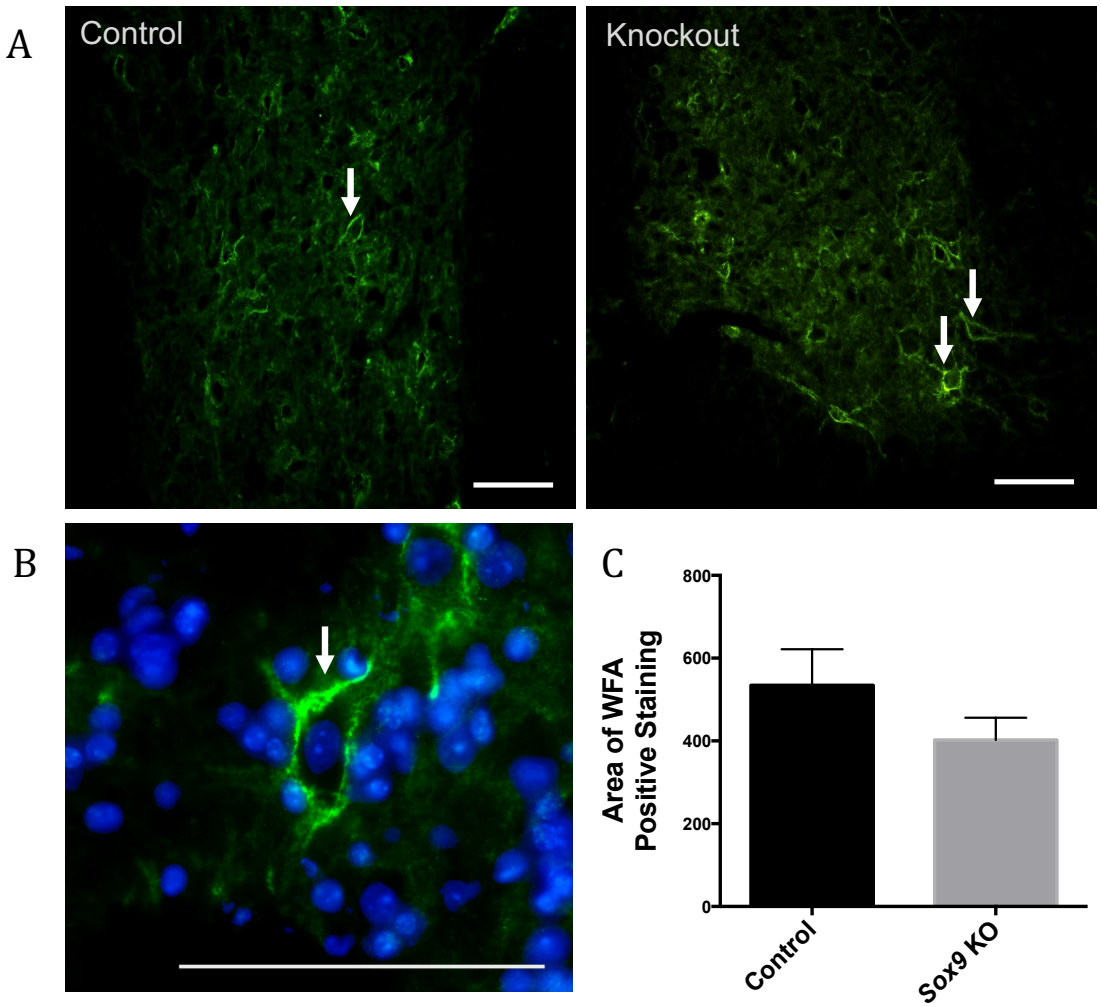
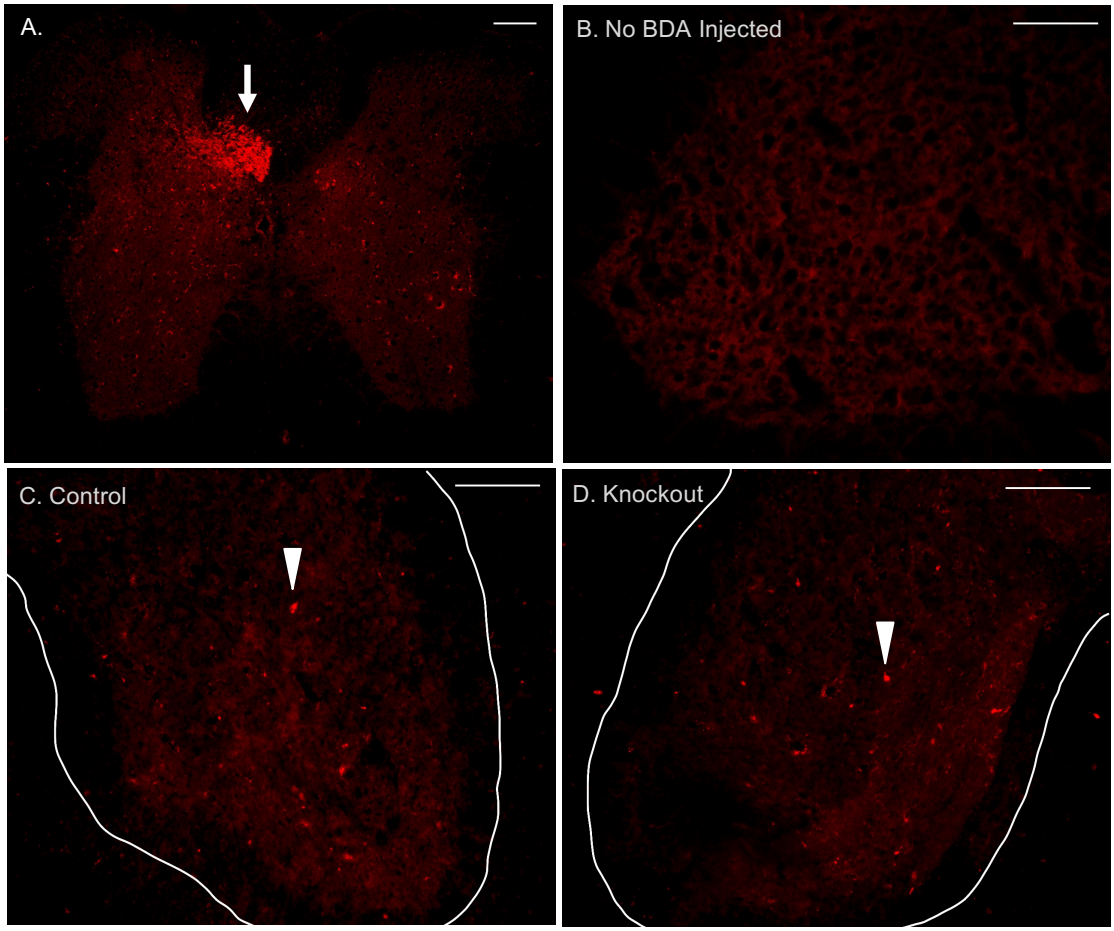


Figure 5. WFA staining of perineuronal nets below the lesion in the ventral horns of control and *Sox9* conditional knockout mice. Arrows point to PNNs. There is no difference in the level of perineuronal nets between *Sox9* conditional KO and control mice. A) Representative photomicrograph of WFA staining in the ventral horn of control and *Sox9* KO mice. Scale bar= 100 μM. B) Photomicrograph of perineuronal net surrounding a neuron. Blue is DAPI staining, scale bar= 100 μM. C) Quantification of WFA demonstrating no difference in PNN levels between control and *Sox9* KO mice. ($p \geq 0.05$, Two-tailed student's t test; n= 9).

3.4 *Sox9* conditional knockout mice do not have increased levels of corticospinal tract fibres

It is possible that *Sox9* ablation 30 days after SCI opens a window of opportunity for neuroplasticity to occur in the injured spinal cord by decreasing levels of CSPGs. In order to assess whether ablating *Sox9* 30 days post-SCI results in increased neuroplasticity, levels of BDA labelled corticospinal tract fibres were quantified. BDA was injected into the mouse hind limb motor cortex 14 weeks post-SCI to label corticospinal tract fibres. Two weeks later, lumbar cross-sections caudal to the lesion were stained for BDA. No differences in the number of BDA-labeled corticospinal tract fibres were found between *Sox9* KO and control mice (Figure 6).


A.
B. No BDA Injected
C. Control
D. Knockout

E

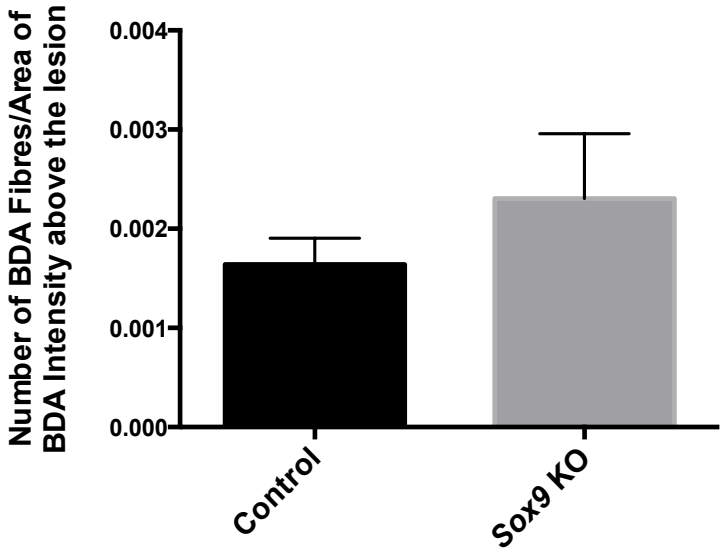

Number of BDA Fibres/Area of BDA Intensity above the lesion
0.004
0.003
0.002
0.001
0.000
Control
Sox9 KO

Figure 6. BDA-labeled corticospinal tract fibres below the lesion in control and *Sox9* conditional knockout mice. There is no difference in the number of BDA fibres between *Sox9* conditional KO and control mice. A) Representative photomicrograph of BDA staining at T10 rostral to the lesion which was used to assess labelling efficiency. Arrow shows the corticospinal tract bundle. B) Negative staining of an animal that did not have BDA injection into the motor cortex. Lack of BDA puncta can be noted. C/D) Representative photomicrograph of BDA staining in the ventral horn (outlined) of control and *Sox9* KO mice. Arrowheads show BDA puncta. Scale bar= 100 μM. E) Quantification of BDA demonstrating no difference between control and *Sox9* KO mice. ($p \geq 0.05$, Two tailed student's t-test; n= 8 control and n= 7 KO).

3.5 *Sox9* conditional knockout mice do not have improved locomotor recovery

My next question was whether ablating *Sox9* weeks after injury would lead to improved locomotor recovery. Locomotor recovery was evaluated in *Sox9* KO and control mice after an L1 SCI by assessing hindlimb function using the BMS. The first BMS score was given 24 hours following SCI and mice with a score greater than 0.5 were excluded from the study. Mice that were kept in the study had a score of 0 or 0.5, translating to completely paralyzed hindlimbs or only slight ankle movement (less than 90°) in one hindlimb (Basso et al., 2006). The BMS was then carried out once a week for 14 weeks. Both control and *Sox9* KO mice showed a gradual significant improvement in locomotor recovery with respect to time (Figure 7). Both groups reached a plateau at 42 days post-SCI (Figure 7). Control mice attained a score of 3.11 ± 0.33 at 14 weeks (98 days) post-SCI, a score which represents an ability to plantar place paws with or without weight support OR dorsal step but not plantar step (Figure 7; Basso et al., 2006). *Sox9* KO mice attained a score of 2.61 ± 0.29 which represents an ability to extensively move the ankle of one hindlimb, and plantar place the other hindlimb with or without weight support OR dorsal step but not plantar step that hindlimb (Basso et al., 2006).

After sacrificing the animals and performing lesion location analysis it was noted that the lesion was more caudal than expected and there was concern that a lesion on the lumbar enlargement would directly injure neurons involved in hindlimb movement. This is because the lumbar enlargement is the site of many motor neuron cell bodies, which supply the lower limbs. Thus, any neuroplasticity occurring below the lesion may not be reflected using the BMS. Because of this, I decided to perform a T9 injury on a new subset of mice (n= 10 KO and n=13 control) and assess hindlimb recovery using the BMS

(Figure 8). These mice demonstrated the same pattern of locomotor improvement with respect to time, and plateaued at approximately 28 days post-SCI. The location of the lesion did not change previous results; *Sox9* KO showed no significant difference in hindlimb movement compared to control mice (Figure 8).

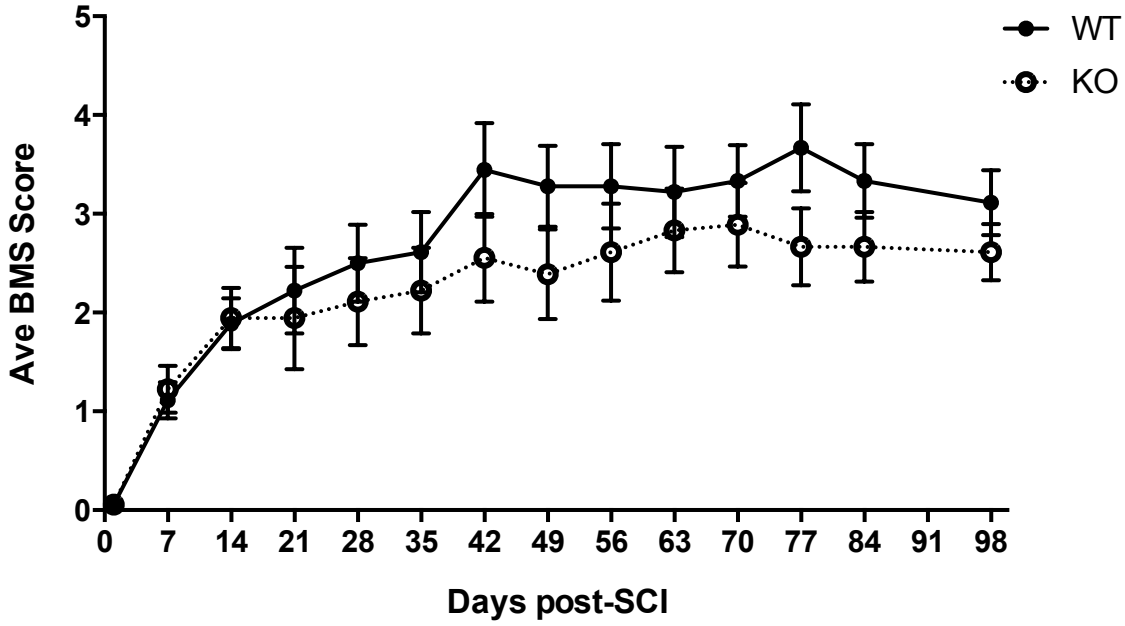


Figure 7. Basso Mouse Scale for control and *Sox9* conditional knockout mice following L1 SCI. Both control and *Sox9* conditional knockout mice display hindlimb paralysis immediately following SCI. Both groups show significant locomotor recovery with respect to time ($p < 0.0001$, Two-way repeated measures ANOVA, n=9). There were no significant differences in hindlimb function at 98 days post-SCI between control and *Sox9* conditional knockout mice. ($p \geq 0.05$, Two-way repeated measures ANOVA; n=9).

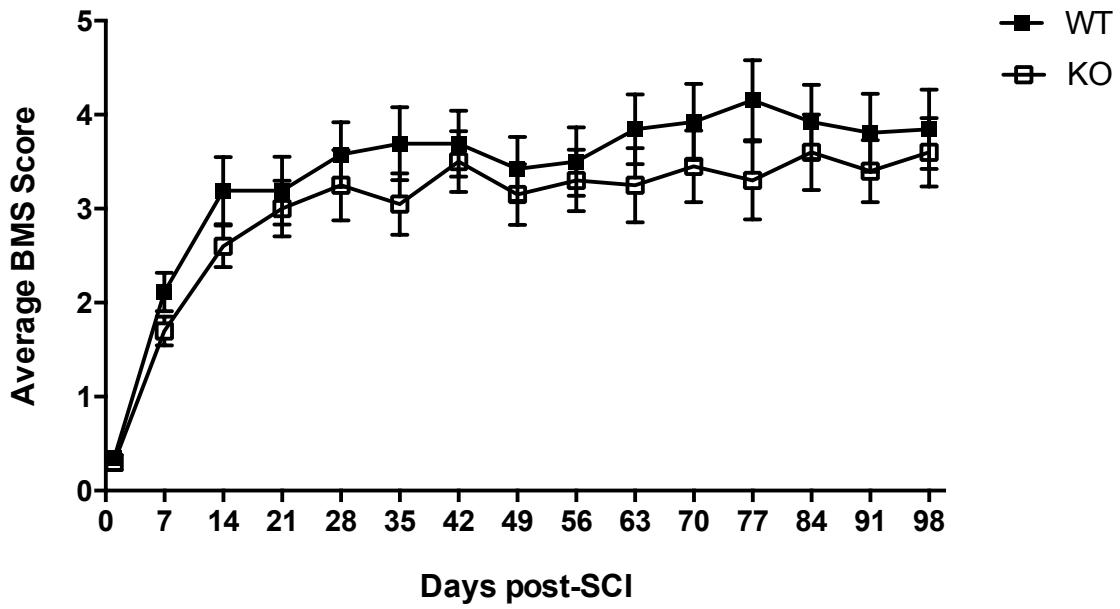


Figure 8. Basso Mouse Scale for control and *Sox9* conditional knockout mice following T9 SCI. Both control and *Sox9* conditional knockout mice display hindlimb paralysis immediately following SCI. Both groups show significant locomotor recovery with respect to time ($p < 0.0001$, Two-way repeated measures ANOVA, n=13 control; n=10 KO). There are no significant differences in hindlimb function at 98 days post-SCI between control and *Sox9* conditional knockout mice. ($p \geq 0.05$, Two-way repeated measures ANOVA; n=13 control and n=10 KO).

Chapter 4: Discussion

There are currently about 85,500 people living with SCI in Canada (Noonan et al., 2012). The majority of individuals with SCI have longstanding disease and therefore it is important to investigate whether a treatment for SCI can be effective when administered at chronic time-points. In addition, knowing if a treatment is effective at later time-points can help define a treatment window for acute SCI. We have shown that ablating *Sox9* just prior to SCI leads to decreased CSPG levels, increased reactive sprouting, and improved locomotor recovery in mice (McKillop et al., 2013; McKillop et al., 2016). CSPG-rich PNNs are a major impediment to neuroplasticity and are known to limit recovery after SCI (Sorg et al., 2016). SOX9 up-regulates a battery of genes necessary for CSPG production and thus, ablation of *Sox9* can decrease CSPG levels and consequently reduce their inhibitory effect on axonal sprouting (Gris et al., 2007; McKillop et al., 2013).

My study focused on modifying the molecular environment of the spinal cord to make it more permissive to axonal sprouting. I aimed to decrease PNNs in a chronic model of SCI by ablating the expression of *Sox9* weeks after injury, and investigating whether *Sox9* ablation can increase reactive sprouting and improve locomotor recovery. I used a tamoxifen inducible conditional *Sox9* KO. The first question I addressed was how long following the onset of tamoxifen administration does it take for *Sox9* expression to be significantly reduced. To answer this question, I measured levels of mRNA at the lesion (L1) and caudal to the lesion (rest of the lumbar spinal cord) at 0, 3, 6, 9, and 14 days after the start of tamoxifen administration. I found that 9 days after the onset of tamoxifen treatment, *Sox9* mRNA levels were significantly reduced in *Sox9$^{f/f}$;Cre* mice

versus the *Sox9*$^{f/f}$ controls both at and below the lesion. Because tamoxifen treatment was initiated 3 weeks after SCI, this means that *Sox9* mRNA was ablated 30 days post-injury.

The next question addressed was whether ablating *Sox9* 30 days post-injury would effectively decrease CSPG levels and PNN matrix. Messenger RNA levels of two individual CSPGs, neurocan and aggrecan, were quantified. Neurocan was significantly decreased in *Sox9* KO mice compared to controls at 6 and 14 days following the onset of tamoxifen administration, while no significant differences were seen in aggrecan levels. Previous work in my laboratory using both siRNA and tamoxifen inducible KO of *Sox9* in astrocyte cell culture has shown that SOX9 regulates expression of neurocan and aggrecan (Gris et al., 2007; McKillop et al., 2013). We have also shown that ablating *Sox9* prior to SCI in mice decreases expression of these CSPGs at the lesion (McKillop et al., 2016). Therefore, I predicted that expression of both neurocan and aggrecan would be decreased following delayed *Sox9* ablation. It is possible that I did not see changes in aggrecan levels because expression of this CSPG is known to drastically decrease following SCI and remains at very low levels at 28 days post-injury, thus detecting a change in expression when protein levels are already low can be difficult (Andrews et al., 2012). This decrease in aggrecan is supported by my study which demonstrated that expression levels of aggrecan at 3 weeks post-SCI were approximately 30% that of neurocan expression.

Two other genes known to be regulated by SOX9 are GFAP and Col4a1 (Gris et al., 2007). GFAP is the major intermediate filament in differentiated astrocytes (Eng, 1985) and is a marker for astrogliosis (Eng and Ghirnikar, 1994). Col4a1 is a protein component of collagen 4, which is present in the lesion epicenter (Klapka and Muller,

2006). Expression of these genes was assessed in *Sox9* KO and control mice after tamoxifen treatment. GFAP expression was reduced at 14 days following tamoxifen treatment. This decreased expression was expected as *Sox9* levels were reduced by day 9. Hypertrophic astrocytes play a big role in glial scar formation after SCI by secreting CSPGs (Silver and Miller, 2004). Attenuating astrogliosis through *Sox9* ablation can decrease CSPGs and their inhibitory effect on axon outgrowth. No difference in Col4a1 expression was found between *Sox9* KO and control mice. We have shown in a previous study that *Sox9* ablation prior to SCI in mice reduces levels of Col4a1 (McKillop et al., 2013). Thus, in chronic phases of SCI, expression of Col4a1 is no longer regulated by SOX9 activity as it is in the acute phase.

At 16 weeks post-SCI, the lesion and lumbar area of the cord caudal to the lesion were stained with WFA to quantify PNN matrix. No significant differences were found between the *Sox9* KO mice and controls. These results demonstrate that ablating *Sox9* 30 days post-SCI is too late to decrease levels of PNN matrix in the spinal cord. The PNN matrix is composed of many different CSPGs including neurocan, aggrecan, brevican, and versican (Deepa et al., 2006). Decreased levels of just one type of CSPG is likely not enough to reduce WFA staining as there are other CSPGs in PNNs for WFA to bind to. WFA allows for visualization of PNNs as it binds to the N-acetylgalactosamines beta 1 (GalNAc beta 1-3 Gal) residues on CSPGs (Hilbig et al., 2001). Aggrecan is known to contain around 100 GAG chains, while other CSPGs including neurocan and brevican contain as little as 0-5 (Siebert et al., 2014). Due to the relative amount of GAG sidechains on CSPGs, it is likely that WFA binding is more indicative of aggrecan, containing a high number of GAG sidechains, than other CSPGs. Thus, I may not have

found differences in WFA staining because aggrecan expression levels were not reduced in *Sox9* KOs.

In a previous study, we ablated *Sox9* just prior to SCI and found decreases in PNN levels, increased reactive sprouting and improved locomotor recovery at 14 weeks post-SCI. Why does ablating *Sox9* at a more chronic time-point, 30 days post-injury, not have the same effect? The most likely explanation is that decreases in CSPG mRNA levels achieved by *Sox9* KO, did not translate into decreased CSPG protein content within the PNN structure. It has been shown that CSPGs in PNNs have a very slow turnover rate (Tsien, 2013; Zimmermann and Dours-Zimmermann, 2008). Margolis et al. (1975) used labeled threonine to calculate the half-life of glycoproteins in the brain and threonine radioactivity showed a biphasic decay in glycoproteins with half-lives of 13 and 38 days. However, they did not observe any incorporation of threonine into glycosaminoglycans and could not measure the half-life of protein in chondroitin sulfate (Margolis et al., 1975). This suggests that the turnover of CSPGs in PNNs is very slow (Tsien, 2013). Matrix metalloproteinases (MMPs) are endogenous endopeptidases that digest PNNs (Tsien, 2013). After spinal cord injury in mice, MMP-9 activity peaks at 24 hours, is greatly reduced at 48 hours, and is all but absent by 7 days post-SCI (Noble et al., 2002). This means that CSPGs are being degraded by MMPs shortly after injury. We have shown that SOX9 levels increase 12-fold at 12 hours post-SCI (Gris et al., 2007). This increased SOX9 expression leads to increases in C4ST, XT-1 and CSPG core proteins neurocan, brevican and aggrecan 1 week after injury. Thus, the breakdown of CSPGs by MMP activity is compensated for by higher levels of SOX9 activity and increased synthesis of CSPGs. SOX9 ablation just prior to SCI prevents the expression of genes

involved in CSPG production (core proteins and biosynthetic enzymes) and the consequent lack of CSPG synthesis combined with increased degradation by MMPs leads to decreased CSPG levels at the lesion (glial scar) and caudal to the lesion (within PNNs) in *Sox9* KO mice. This decrease makes the molecular environment of the spinal cord more permissive to plasticity, and is why we found increased reactive sprouting and locomotor recovery when we ablated *Sox9* prior to injury. I suggest that in the present study in which *Sox9* is ablated well after MMP activity has ceased to be detectable (after 1 week post-injury), CSPG levels remain unchanged from baseline levels because in the absence of MMP activity CSPG turnover is extremely low. This explanation is in keeping with our prior demonstration that if MMP activity is inhibited concurrently with conditional *Sox9* ablation at the time of SCI, CSPG levels do not decrease nor is there any improvement in locomotor recovery (McKillop et al., 2016). Thus, both the presence of MMPs and *Sox9* ablation is needed to effectively decrease CSPG rich PNNs (Figure 9).

My next question was whether ablating *Sox9* weeks after injury would allow for axonal sprouting to occur. Quantification of BDA puncta in the ventral horns below the level of the lesion demonstrated no differences in labeled corticospinal tract neurons in *Sox9* KO mice versus controls. After discovering that *Sox9* ablation 30 days after SCI does not decrease the amount of PNNs at or below the lesion, it was expected that no neuroplasticity would occur. Without decreased PNNs, the molecular environment of the spinal cord remains inhibitory to axonal sprouting. If PNNs were decreased in *Sox9* KO mice, I would have expected these mice to have increased BDA labeling signifying increased neuroplasticity.

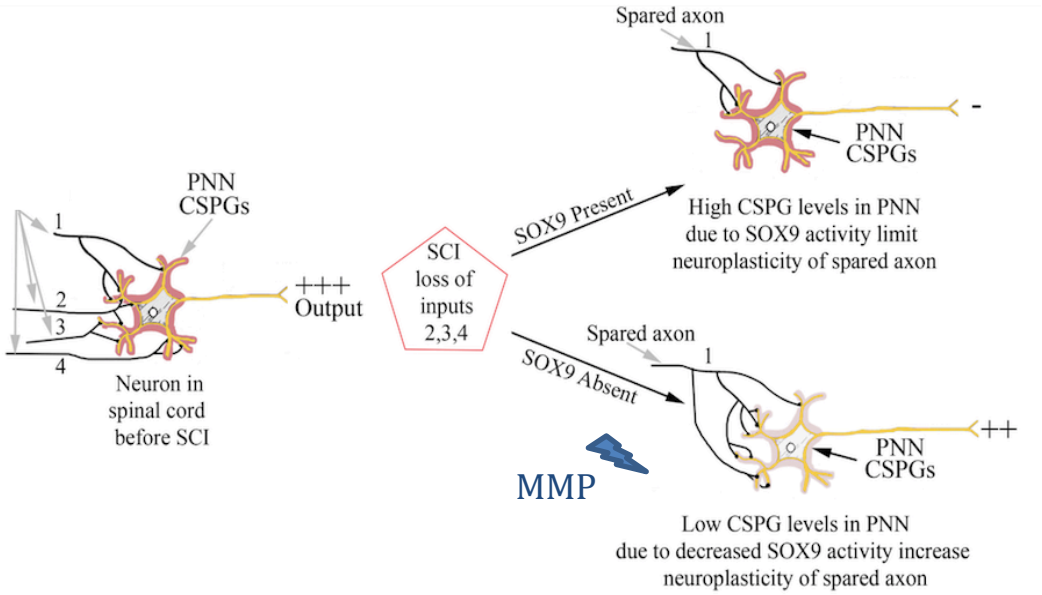


Figure 9. Diagram describing how *Sox9* ablation leads to increased neuroplasticity. Prior to SCI, the neuron illustrated on the left receives many inputs. After SCI, there is a loss of inputs synapsing on the neuron and only inputs from spared axons remain. SOX9 activity increases synthesis of CSPGs which limit neuroplasticity. When no SOX9 is present, there are decreased levels of CSPGs in PNNs, which allows axonal branching to occur from spared fibres to compensate for the loss of synaptic input on neurons. However, this response only occurs in acute stages of SCI because turnover of CSPGs is high due to the increased presence of MMPs. In chronic stages of SCI, MMP levels are not high, turnover of CSPGs is low, and ablation of *Sox9* does not lead to decreases in CSPGs. Therefore, both *Sox9* ablation and the presence of MMPs is needed to decrease PNN CSPGs.

An alternate explanation for why axonal sprouting did not take place could be that neurons did not possess an intrinsic ability for sprouting in chronic stages of SCI. Intrinsic factors would include proteins expressed within neurons that promote axonal growth and sprouting. For example, ß-actin and GAP-43 are two proteins that support axon branching and elongation, respectively (Donnelly et al., 2013). Expression of some of these proteins may be increased shortly after SCI, thus promoting axon sprouting in the acute phases of SCI. It has been found that 1-4 days after injury growth associated protein (GAP) 43 immunoreactivity is increased in axons after mild and moderate compression SCI in rats, but begins to decrease 9 days post-injury (Li et al., 1996). This means that in the first few days after SCI, axon elongation may be supported due to the presence of GAP-43.

Extrinsic factors in the injured spinal cord microenvironment such as those in myelin, could also explain why neuroplasticity did not occur. There are molecules present in myelin that limit neuroplasticity including Nogo, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, netrin, and ephrin (Vourc'h and Andres, 2004; Kempf and Schwab, 2013; Blesch and Tuszynski, 2009). It is possible that in the acute phases of SCI these molecules decrease in expression allowing for sprouting to occur, and return to baseline levels at later time-points. For example, Nogo-A protein and mRNA expression are low 24 hours after SCI in rats, and at their lowest 3 days post-injury (Wang et al., 2015). They then peak at 7 days and drop only slightly 14 days following SCI (Wang et al., 2015). This means that in the acute phases of SCI, inhibition on axon outgrowth by this myelin-associated molecule is low, but increases at later time points making it a greater challenge to induce plasticity later than a week after SCI.

Since neuroplasticity did not increase in *Sox9* KO mice as assessed with BDA, improvements in recovery were not expected to occur. Locomotor recovery was recorded for 14 weeks using the BMS to assess hindlimb function. Both *Sox9* KO mice and controls improved significantly with respect to time. This was expected as spontaneous natural recovery does occur following SCI. However, *Sox9* KO mice did not show greater hindlimb recovery compared to controls.

When the location of the lesion was landmarked, I discovered that the injury was centered at the L1 level whereas I planned for the injury to be located at T9. A contusion injury directly on the lumbar enlargement would physically damage the alpha motor neurons that innervate hindlimb muscles. Thus, any sprouting that occurs onto these motor neurons may not improve hindlimb movement. It is formally possible that I did not find differences in the BMS score, which reflects hindlimb movement, between *Sox9* KO and control mice because of my lesion location. Therefore, I generated another group of spinal cord injured animals possessing a lesion at T9 to re-assess locomotor recovery. It was found that *Sox9* KO mice with a T9 lesion also did not show improved hindlimb recovery compared to controls. Therefore, I can affirm that the lack of improved recovery is due to the absence of sprouting as opposed to neuronal destruction at the lumbar enlargement.

As our laboratory has a research program dedicated to identifying and preclinical testing of SOX9 inhibitors it is interesting to consider what this study says about the likely therapeutic usefulness of SOX9 inhibition after SCI. My findings suggest that if a SOX9 inhibitor is to be administered on its own then it will need to be delivered in the acute period of SCI when MMP activity (and hence CSPG turnover) is high. In the mouse

model of SCI, this period is defined by a period of days (Hansen et al., 2013). However, there are differences in the start and duration of inflammation between rodents and humans, which suggest that a window for the administration of a SOX9 inhibitor may be weeks or months in the human (Hansen et al., 2013). Immunohistochemical analyses of *post mortem* human spinal cords from controls and patients who sustained a SCI reveal that expression of MMP-9 rises progressively from 5 to 24 days post-injury, while MMP-12 experiences an isolated peak of expression at 24 days post-injury (Buss et al., 2007). Based on these results, a SOX9 inhibitor may prove effective in humans when administered up to 24 days following spinal cord injury.

For the treatment for chronic SCI, my results suggest that a method to increase the turnover of CSPGs should be applied before or at the same time as the SOX9 inhibitor. This process may be possible by inducing an acute inflammatory response in the cord, as inflammation has been known to induce MMP production by glial cells, endothelial cells, and leukocytes (Hansen et al., 2013; Buss et al., 2007). Lipopolysacharide (LPS) can be injected to induce an inflammatory response (Rosenberg, 2002). It has been shown that LPS induces the expression of MMP in human monocytes and macrophages, and murine macrophages and astrocytes (Ho et al., 2008; Woo et al., 2004; Lee et al., 2003). With these treatment combinations, CSPGs will be effectively degraded and the body not capable of replenishing their levels due to *Sox9* ablation.

Perhaps the best therapeutic approach to SCI will be strategies that in addition to removing impediments to sprouting also promote growth of axons. Expression of a variety of growth associated proteins and signals are required within the neuron and axon to be able to sense extracellular matrix cues and trophic factors that guide sprouting

(Blesch and Tuszynski, 2009). Thus, an effective treatment for chronic SCI will likely consist of a combinatorial treatment targeting both the axon's ability to grow, and the inhibitory molecular environment of the injured spinal cord. One method that primes axons for sprouting is to increase their activity, which can be accomplished through electrical stimulation or rehabilitation (Onifer et al., 2011). Rehabilitation for spinal cord injured patients is currently the most effective treatment for improving locomotor and sensory recovery (Onifer et al., 2011). It is believed that rehabilitation, as well as electrical stimulation, increase levels of 3'-5'-cyclic adenosine monophosphate (cAMP) and brain-derived neurotrophic factor (BDNF), molecules known to increase neuroplasticity (Onifer et al., 2011). Increased cAMP levels cause upregulation of growth-associated genes in the neuron, while BDNF is a trophic factor that affects synaptic plasticity (Qiu et al., 2002; Ying et al., 2008). Wang et al. (2011) demonstrated the effects of a combination treatment consisting of chondroitinase administration and rehabilitation in rats after a C4 SCI. Rats given the combination treatment performed better at skilled paw reaching than rats given chondroitinase or rehabilitation alone (Wang et al., 2011). Furthermore, the administration of this combination treatment was performed 1 month after SCI, thus showing efficacy in a chronic model of injury. Trophic factors also seem likely as candidates for inclusion in a combination treatment in order to provide a cue for axon outgrowth. Neurotrophin-3 has been shown to increase sprouting of the CST in rats after SCI and is one of a few possible neurotrophins that could be utilized (Schnell et al., 1994). Combinatorial treatments, including those utilizing neurotrophins and cAMP, are actively being researched and have shown success in promoting axonal regeneration (Lu et al., 2004; Pearse et al., 2004; Schnell et al., 1994).

In my study, I set out to find whether ablation of the SOX9 transcription factor in chronic SCI shows the same benefits as in acute ablation. I found that this was not the case. Ablation of *Sox9* 30 days after SCI did not lead to decreased levels of PNNs, increased reparative sprouting, or improved locomotor recovery. I have argued that this lack of effect is due to the slow turnover of CSPGs in the spinal cord and that a future SOX9 inhibitor should be applied with treatments targeting CSPG degradation. It is important to define a therapeutic time window when investigating specific treatments for SCI, as early intervention may not be possible. Physicians must often wait until a patient is stable after experiencing trauma to the cord before a therapy can be administered. Thus, treatments for SCI may have to be applied days after an injury has been sustained. Furthermore, there are many individuals with longstanding SCI who would benefit from treatment applied during chronic phases of injury. Although there are no current treatments for SCI, investigations focused on increasing reparative sprouting may yield promising results.

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Ethics Approval for the Use of Animals on Protocol 2015-004

Western

2015-004::2:
AUP Number: 2015-004
AUP Title: Investigations of CNS Injury and Regenerative Therapies
Yearly Renewal Date: 05/01/2017
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2015-004 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: Schoelier, Marianne
on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 ● FL 519-661-2028
Email: auspc@uwo.ca ● http://www.uwo.ca/animal/website/

Curriculum Vitae

Natalie Ossowski

Education

Master of Science- Neuroscience
The University of Western Ontario
2014-present
Research Supervisor: Dr. Arthur Brown

Bachelor of Medical Sciences- Honors Specialization in Medical Sciences
The University of Western Ontario
2009-2013

Research Honours and Awards

Natural Sciences and Engineering Research Council of Canada (NSERC)
Alexander Graham Bell Canada Graduate Scholarship- Master's Program
2015/09-2016/09

Ontario Graduate Scholarship- Master's Program
(awarded but I declined in order to accept NSERC scholarship)
2015/07

Western Graduate Research Scholarship
2014/09-2016/08

Publications

McKillop WM., York EM., Rubinger L., Liu T., Ossowski NM., Xu K., Hryciw T. and Brown A. (2016). Conditional *Sox9* ablation improves locomotor recovery after spinal cord injury by increasing reactive sprouting. *Exp Neurol.* 283, 1-15.

Research Presentations

Ossowski, N. M., MacMillan, R. A., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2017). *Delayed Sox9 ablation in a mouse model of chronic spinal cord injury.* Robarts Research Retreat, London ON, Canada.

Ossowski, N. M., MacMillan, R. A., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2017). *Delayed Sox9 ablation in a mouse model of chronic spinal cord injury.* Canadian Association for Neuroscience Annual Meeting. Montréal QC, Canada.

Ossowski, N. M., MacMillan, R. A., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2017). *Delayed Sox9 ablation in a mouse model of chronic spinal cord injury.* Combined Canadian Spinal Cord Injury and Ontario Spinal Cord Injury Research Network Meeting. Toronto ON, Canada.

Ossowski, N. M., MacMillan, R. A., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2017). *Delayed Sox9 ablation in a mouse model of chronic spinal cord injury.* Southern Ontario Neuroscience Association Annual Meeting. St. Catherines ON, Canada.

Ossowski, N. M., MacMillan, R. A., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2017). *Investigating locomotor recovery and neuroplasticity in the mouse spinal cord following Sox9 ablation three weeks post injury.* London Health Research Day Annual Meeting. London ON, Canada.

Ossowski, N. M., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2016). *Neuroplasticity in the injured spinal cord following Sox9 ablation three weeks post-injury.* Society for Neuroscience Annual Meeting. San Diego CA, USA.

Ossowski, N. M., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2016 June). *Neuroplasticity and locomotor recovery in the injured mouse spinal cord following Sox9 ablation at three weeks post injury.* Robarts Research Retreat, London ON, Canada.

Ossowski, N. M., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2016 May). *Neuroplasticity and locomotor recovery in the injured mouse spinal cord following Sox9 ablation at three weeks post injury.* Neuroscience Research Day, London ON, Canada.

Ossowski, N. M., Geremia, N. M., Hryciw, T., Xu, K., and Brown, A. (2016 May). *Opening the Window of Plasticity in the Injured Spinal Cord*. Oral presentation at 3 Minute Thesis Competition- Schulich School of Medicine and Dentistry Heat, London ON, Canada.

Related Employment and Volunteer Experience

Graduate Teaching Assistant- The University of Western Ontario
Course: Anatomy and Cell Biology 3319- Systemic Human Anatomy
2014/09-2015/04

Neuroscience Graduate Student Representative and Schulich Graduate Student Council Member
The University of Western Ontario
2015-2017

Laboratory Research Volunteer
The University of Western Ontario
Laboratory of Dr. Andrew Leask, PhD
Schulich School of Medicine & Dentistry, Department of Physiology & Pharmacology
2013/09-2014/04

Laboratory Research Volunteer
The University of Western Ontario
Laboratory of Dr. David Litchfield, PhD
Schulich School of Medicine & Dentistry, Department of Biochemistry
2012/10-2013/04