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Investigation of Sox9 Ablation on Neural Stem Cell Behaviour after Spinal Cord Injury

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INVESTIGATION OF *SOX9* ABLATION ON NEURAL STEM CELL

BEHAVIOUR AFTER SPINAL CORD INJURY

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

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The School of Graduate and Postdoctoral Studies

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Abstract

After spinal cord injury neural stem cells are activated to proliferate and differentiate primarily into astrocytes, but are unable to replace lost neurons or aid in neurological recovery. Recent research shows that the transcription factor *Sox9* promotes gliogenesis while inhibiting neurogenesis, and that *Sox9* ablation causes improved recovery after spinal cord injury. The purpose of this study was to determine how *Sox9* ablation alters neural stem cell behaviour after spinal cord injury and whether it leads to neurological improvements. We used BrdU and YFP to label and track neural stem cells and a neural stem cell-specific *Sox9* knockout mouse model to isolate their role in improving recovery. We found that although *Sox9* ablation reduced neural stem cell proliferation and gliogenesis while increasing neurogenesis, the effects were too small to have a significant impact on neurological recovery. Thus, additional factors must be altered in addition to *Sox9* ablation for significant results.

Keywords

"There is no end to education. It is not that you read a book, pass an examination, and finish with education. The whole of life, from the moment you are born to the moment you die, is a process of learning".

- Jiddu Krishnamurti

"Nobody climbs mountains for scientific reasons. Science is used to raise money for the expeditions, but you really climb for the hell of it".

- Edmund Hillary

"Outside of a dog, a book is a man's best friend. Inside of a dog it's too dark to read".

- Groucho Marx

Dedications

There are a few important people in my life I want to dedicate this thesis to. I want to thank my girlfriend, Jessica Hann, who I have lived with throughout my Master's degree. Coming home to you every day reminded me that there is life outside of the lab. Your loving support made it all possible. Also, to our dog, Buddy, you could always cheer me up when experiments failed.

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List of Abbreviations

APC	adenomatous polyposis coli protein	**PFA**	paraformaldehyde
B-Gal	beta-galactosidase	**PTP**	protein tyrosine phosphatase
BMS	basso mouse scale	**RNA**	ribonucleic acid
BrdU	5-bromo-2'-deoxyuridine	**SCI**	spinal cord injury
CMV	cytomegalovirus	**TGF-β1**	transforming growth factor beta 1
CNS	central nervous system		
Cre-ER	Cre-mutated estrogen receptor	**XT-I**	xylosyltransferase-I
CSPG	chondroitin sulfate proteoglycans	**XT-II**	xylosyltransferase-II
DAPI	4',6-diamidino-2-phenylindole	**YFP**	yellow fluorescent protein
DCX	doublecortin		
DNA	deoxyribonucleic acid		
dpc	days post conception		
GFAP	glial fibrillary acidic protein		
GSK-3β	glycogen synthase kinase 3 beta		
HCl	hydrochloric acid		
IL-6	interleukin 6		
NeuN	neuronal nuclei		
NSC	neural stem cell		
miRNA	micro RNA		
mRNA	messenger RNA		
PBS	phosphate buffered saline		
PCR	polymerase chain reaction		

Chapter 1:

Introduction:

1.1 Spinal cord injury prevalence

Spinal cord injury is a Central Nervous System (CNS) injury often causing paralysis and other serious problems that affects thousands of Canadians each year (Noonan, Fingas et al. 2012). Spinal cord injuries are classified into two groups, traumatic and non-traumatic spinal cord injuries. Traumatic spinal cord injuries are those with an initial impact that damages the spinal cord, such as injuries caused by motor vehicle accidents or sports injuries. Non-traumatic spinal cord injuries include those that are acquired more slowly over time such as those caused by infections, tumours, or spondylosis (a degenerative osteoarthritis of the joints in the vertebral column) (Noonan, Fingas et al. 2012). Using data from 2010 there are an estimated 86, 000 people currently living with either traumatic or non-traumatic spinal cord injury in Canada alone, with traumatic spinal cord injury accounting for 51% of the cases (Noonan, Fingas et al. 2012). It is estimated that 37, 000 of the Canadians living with spinal cord injury are suffering from tetraplegia, a paralysis leading to a partial or total loss of use of all of their limbs and their torso, and that 48, 000 are living with paraplegia, an impairment in just their lower limbs. Annually the costs for traumatic spinal cord injuries in Canada are approximately $3.6 billion (2013). This seriously debilitating disease reduces the quality of life for millions of people worldwide (Wyndaele and Wyndaele 2006) and there are currently no effective treatments for people suffering from spinal cord injuries (Tator

2006). However, current research is looking into potential therapies that target the processes that worsen the injury and inhibit recovery.

1.2 Secondary processes after traumatic spinal cord injury

After a traumatic spinal cord injury there are many secondary processes that exacerbate the initial trauma and impede recovery (Oyinbo 2011). A primary spinal cord injury is unpredictable and the only possible treatments are preventative measures; however, secondary injury may be targeted with specific treatments in order to reduce ongoing damage to the spinal cord and hopefully lead to recovery from the initial injury (Tohda and Kuboyama 2011). For the majority of patients it is the extent of this damaging secondary injury that can predict their long-term morbidity (Dumont, Okonkwo et al. 2001).

Inflammation underlies much of the secondary injury after spinal cord injury (Oyinbo 2011). Immediately after spinal cord injury immune cells invade the injury site and release pro-inflammatory cytokines which activate inflammation that can persist for several weeks or months following injury (Liverman 2005; Fehlings and Nguyen 2010). This overactive inflammatory response can lead to tissue damage or neuron death. Oligodendrocytes appear to be particularly vulnerable after spinal cord injury showing significant apoptosis/cell death as quickly as 15 minutes after injury (Grossman, Rosenberg et al. 2001) and continuing up to one week after injury (Casha, Yu et al. 2001). Oligodendrocyte death leads to a loss of myelin around axons leaving them exposed to the damaging environment after injury, further increasing neuron loss.

Astrocytes become activated after spinal cord injury and migrate to the injury site where they form the glial scar (Fawcett and Asher 1999). This scar acts as a barrier to descending axons preventing regrowth and reconnection below the level of the lesion (Silver and Miller 2004). The scar also prevents oligodendrocytes from reaching axons demyelinated by secondary injury (Fok-Seang, Mathews et al. 1995). The glial scar is largely produced by activated astrocytes that release many growth-inhibiting molecules that act as chemical barriers to axonal regrowth (Bahr, Przyrembel et al. 1995; Davies, Goucher et al. 1999). For example, reactive astrocytes within the glial scar produce chondroitin sulfate proteoglycans that have been shown *in vitro* to block neurite outgrowth (McKeon, Schreiber et al. 1991; Zuo, Neubauer et al. 1998). Myelin and myelin associated proteins such as myelin associated glycoprotein, Nogo-A, and oligodendrocyte myelin glycoprotein similarly act as a chemical barrier to axonal regeneration (Caroni and Schwab 1988; Schwab, Failli et al. 2005; Yiu and He 2006; Akbik, Cafferty et al. 2012). These regeneration-inhibiting processes combine to severely increase the damage to the spinal cord in the days and weeks following the primary injury and create a growth inhibiting environment preventing the recovery of function below the injury.

The lack of recovery and regeneration after central nervous system injury is contrasted by recovery of the peripheral nervous system from injury (Tucker and Mearow 2008). Previous research has shown that not only can the peripheral nervous system regrow and recover after injury, but the exact same neurons that are incapable of regrowth in the central nervous system are capable of regeneration when presented with the environment of the peripheral nervous system (David and Aguayo 1981). This

demonstrates that the regrowth of central nervous system neurons past an injury is possible, but that the environment of the injured central nervous system needs to be changed to promote recovery. Thus one strategy to improve recovery after spinal cord injury is to attempt to alter the microenvironment of the injured central nervous system from one that inhibits regeneration and recovery to one that promotes and encourages recovery. Recovery from spinal cord injury is also hampered by neuronal and glial cell loss (Liu, Xu et al. 1997). Thus a second strategy to improve recovery from spinal cord injury is to focus on cell replacement (Barnabe-Heider and Frisen 2008). Neural stem cell therapies provide a unique opportunity for the treatment of spinal cord injury as they are natural vehicles for cell replacement strategies in the injured central nervous system and also have the ability to alter the injured central nervous system microenvironment (Meletis, Barnabe-Heider et al. 2008).

1.3 Neural stem cell properties

A neural stem cell has the basic properties of all stem cells: the ability to self-renew, and the ability to differentiate into different cell types (Johansson, Momma et al. 1999). Neural stem cells are capable of producing three different cell types which all reside in the central nervous system (Johansson, Momma et al. 1999) and are shown in Figure 1. These three cell types are neurons, and the glial cells astrocytes and oligodendrocytes. During development neural stem cells are responsible for producing progenitor cells that give rise to all three of these cell types in the central nervous system (Qian, Shen et al. 2000; He, Ingraham et al. 2001). After development neural stem cells become quiescent save for only a couple of areas in the brain (including the

Figure 1: The neural stem cell lineage (cell-specific markers expressed by each cell type are shown in brackets). Neural stem cells are capable of self-renewal and of producing the three primary cell types in the central nervous system; neurons, oligodendrocytes, and astrocytes. Neural stem cells are able to differentiate into glial progenitor cells, which may differentiate into the glial cells astrocytes or oligodendrocytes, as well as neuroblasts which may mature into adult neurons. Image modified from University of California, San Francisco, Division of Neonatology website (2009).

Self-renewing neural stem cells (Nestin, SOX9)

Early progenitor cells (Nestin, SOX9)

Neuroblasts (DCX)

Glial progenitor cells (SOX9)

Neurons (NeuN)

Oligodendrocytes (APC, SOX9)

Astrocytes (GFAP, SOX9)
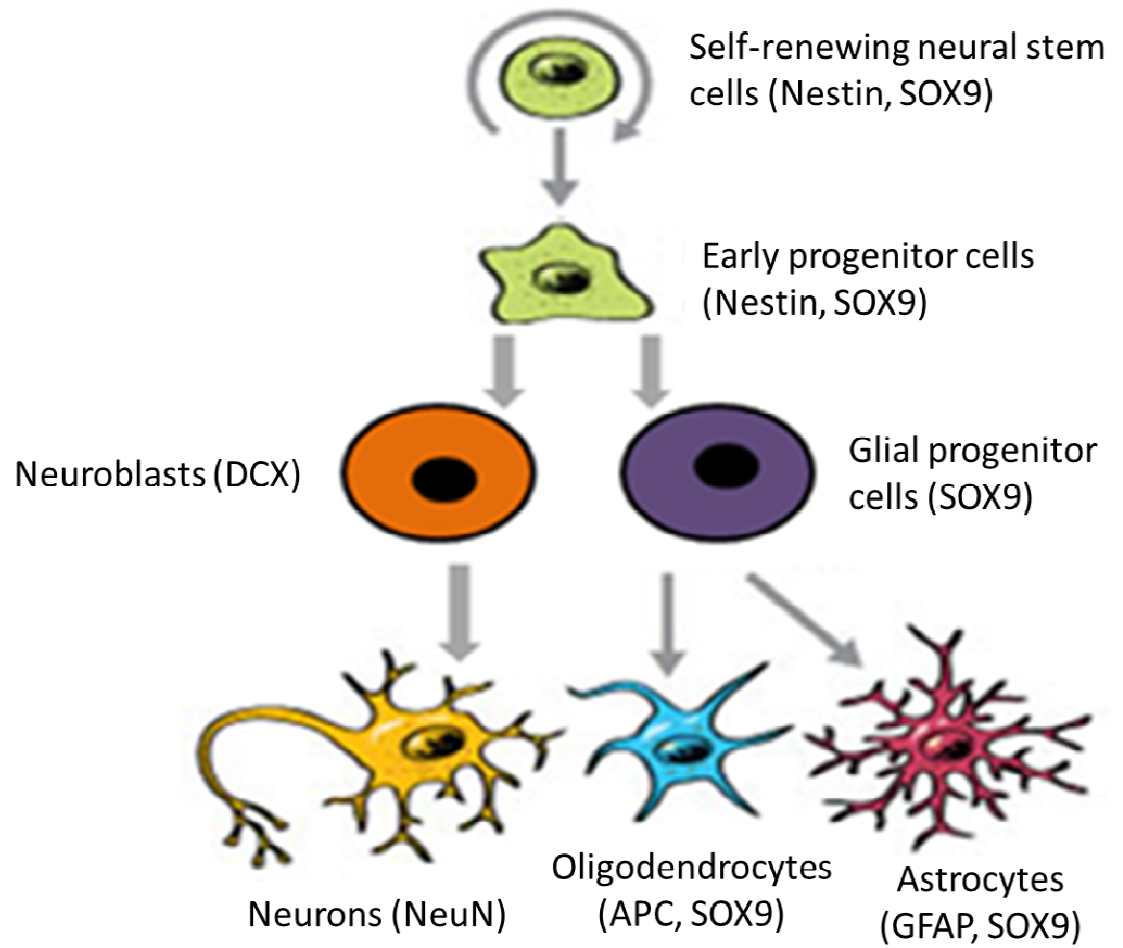
hippocampus, olfactory bulb, subcallosal zone, and the cerebellum) that continue to produce neurons throughout life (De Filippis and Binda 2012). Neural stem cells reside in the ependymal layer of the spinal cord, the layer of cells directly surrounding the cerebrospinal fluid-filled central canal (Johansson, Momma et al. 1999; Horner, Power et al. 2000; Meletis, Barnabe-Heider et al. 2008). Although neural stem cells reside in the spinal cord, many researchers have attempted the transplantation of exogenous neural stem cells as a potential treatment for spinal cord injuries.

1.4 Exogenous neural stem cell transplantation therapies

Due to their potential to produce new neurons and oligodendrocytes, many researchers have attempted to inject neural stem cells into an injured spinal cord as a potential method to replace lost cells and allow new neurons to form connections across the injury site to regain function (Li and Lepski 2013). Some studies using this transplantation method have found that although many of the injected cells survive transplantation, the majority differentiate into astrocytes with either a very small amount or no new neurons being produced (Cao, Zhang et al. 2001; Webber, Bradbury et al. 2007). Webber's study also showed that the transplantation was unable to improve functional outcomes using several behavioural tests. The authors suggest that the injured and uninjured spinal cord microenvironment only supports glial as opposed to neuronal differentiation of neural stem cells as neural stem cells produce neurons when placed into hippocampal regions in the brain, but not when transplanted into uninjured spinal cords where they are only able to generate glial cells (Shihabuddin, Horner et al. 2000). Recently, it has been shown that treating neural stem cells prior to implantation has been

successful at promoting the production of neurons and oligodendrocytes in the injured

central nervous system (Li and Lepski 2013). For example, researchers have used various

strategies such as priming the stem cells toward a neural lineage by growing them in

culture with specific molecules until many of the cells have a neural phenotype before

their transplantation (Harper, Krishnan et al. 2004; Tarasenko, Gao et al. 2007). Others

used a similar strategy but instead primed the stem cells toward an oligodendrocyte

lineage (Liu, Qu et al. 2000). Other strategies involved modifying neural stem cells to

express the Olig2 transcription factor which regulates oligodendrocyte development to

increase oligodendrocyte numbers and white matter sparing (Hwang, Kim et al. 2009),

and the injection of exogenous neurotrophic factors with the stem cells (Bregman,

Coumans et al. 2002). These more successful attempts to restore neuron and

oligodendrocyte numbers by altering the behaviour and/or genetics of the neural stem

cells may be promising for improving recovery, however it does not change the fact that

these strategies all rely on allografts and thus if ever used clinically would be faced with

problems of immunological rejection (Preynat-Seauve, de Rham et al. 2009).

Furthermore, as with any transplantation strategy the introduction of highly proliferative

cells into a patient also poses the risk of tumorigenesis (Gordeeva 2011). One study

showed that the transplantation of human embryonic stem cell derived neural stem cells

into injured and uninjured spinal cords of rats produced teratomas in all of the animals

(Sundberg, Andersson et al. 2011).

A safer and more efficient strategy may be to alter the behaviour of the

endogenous neural stem cells with the goal of generating increased numbers of neurons

and oligodendrocytes and fewer astrocytes. An increased number of neurons might form

new connections that bridge the lesion in the injured cord and an increased number of oligodendrocytes might help remyelinate denuded axons (Liu, Qu et al. 2000). At the same time decreasing astrocyte numbers could potentially reduce the levels of scaring and of growth inhibiting proteins in the injured cord (McKillop, Dragan et al. 2013).

1.5 Endogenous neural stem cells after spinal cord injury

An injury to the spinal cord activates neural stem cells and causes them to proliferate rapidly (Johansson, Momma et al. 1999; Lytle and Wrathall 2007; Barnabe-Heider, Goritz et al. 2010). The Frisen lab was able to show that neural stem cells and their progeny migrate to the lesion site and contribute to the glial scar with the use of two mouse strains: 1) a strain in which the Cre-estrogen receptor (Cre-ER) recombinase is expressed under the control of the *FoxJ1* promoter providing neural stem cell specific Cre-ER expression and 2) a Cre reporter mouse line in which beta-galactosidase (βGal) RNA transcription is under the control of the *ROSA* locus but protein expression requires a Cre excision event (Meletis, Barnabe-Heider et al. 2008). In the *FoxJ1*-Cre-ER mice the Cre recombinase is fused to a mutated form of the estrogen receptor. In the *ROSA*-βGal mouse line β-galactosidase mRNA is expressed in all cells but not translated owing to the presence of a loxP-flanked strong stop sequence at the 5'-end of the message. The administration of tamoxifen to the *FoxJ1*-Cre-ER;*ROSA*-βGal mice induces the translocation of the Cre-ER fusion protein to the nucleus of the cell where it excises DNA between loxP sites, removing the stop signal and allowing for β-galactosidase protein expression. In *FoxJ1*-Cre-ER;*ROSA*-βGal mice only the neural stem cells in the spinal cord express Cre-ER and thus, following tamoxifen administration, all neural stem cells

and their progeny will express β-galactosidase for the rest of that animal's life. Using this mouse model these researchers found that after spinal cord transection, neural stem cells migrate into the primary injury site and remain in the lesion for up to ten months. The Frisen laboratory demonstrated that the majority of these neural stem cells did not differentiate into a mature phenotype. However, the neural stem cell progeny that did express mature cell markers were mostly astrocytes and a few oligodendrocytes. Therefore endogenous neural stem cells play an active role in the response to a spinal cord injury. However, they fail to produce new neurons after an injury despite being activated (Sabelstrom, Stenudd et al. 2013). Thus it appears that spinal cord injury can activate neural stem cells to proliferate and migrate into the lesion but does not allow new neurons to be generated. Since neural stem cells taken from an adult spinal cord can give rise to neurons when moved to the dentate gyrus of the hippocampus, it would appear that it is the environment of the spinal cord that inhibits their potential for neuronal differentiation (Shihabuddin, Horner et al. 2000). Theoretically, there might be two possible strategies to promote neurogenesis after injury. One would be to change the injured spinal cord microenvironment to promote the neuronal differentiation of neural stem cells. The other would be to alter the genetics of the neural stem cells so that they possess a bias toward neuronal differentiation. This second approach is the subject of this thesis and discussed below.

1.6 The transcription factor *Sox9* directs neural stem cell differentiation down a glial lineage

Sox9 is a part of the *Sox* family of transcription factors which consists of 20 different genes all with a high mobility group (HMG-box) DNA binding domain with a high degree of sequence homology to SRY (testis-determining factor) (Moniot, Declosmenil et al. 2009). Knocking out *Sox9* in the developing central nervous system causes perinatal lethality (Bi, Huang et al. 2001). The nervous system of *Sox9* knockout mice shows decreased numbers of oligodendrocyte progenitors and astrocytes, and an increased number of motor neurons (Stolt, Lommes et al. 2003). At 14.5 days post conception (dpc) *Sox9* deficient embryos only had 25% as many oligodendrocyte progenitors as wild-type mice and virtually no astrocytes in their spinal cords. At 12.5 dpc *Sox9* deficient embryos showed a 30% increase in motor neurons. This suggests that during development *Sox9* promotes the production of glial cells (oligodendrocytes and astrocytes) and inhibits the production of neurons from neural stem cells.

In addition to promoting gliogenesis, *Sox9* has also been shown to be required to maintain fully functioning neural stem cells. (Scott, Wynn et al. 2010). These researchers studied the initial formation of neural stem cells. To determine if neural stem cells were present they tested the cells in the central nervous system for the two basic properties of stem cells. They tested if the cells could proliferate and self-renew by their ability to form neurospheres. They also tested if the cells had multipotentiality (the ability to form neurons, astrocytes, and oligodendrocytes). These researchers found that in both mouse and chick embryos, neural stem cells are first present just after SOX9 expression is up-regulated. When SOX9 expression was induced at earlier time points (before the onset of

neural stem cells) neurosphere forming neural stem cells were produced. When *Sox9* was knocked out, the cells from the central nervous system produced significantly less neurospheres, only about 10% as many as the wild-type mice. This suggested that *Sox9* is necessary for neural stem cell proliferation and/or survival. They also found that both in the developing central nervous system and in adult mice reduced SOX9 expression decreased the production of astrocytes, oligodendrocyte precursors, and neurons from neural stem cells, while increasing neuroblasts production. These findings supported the results from the *Sox9* knockout paper (Stolt, Lommes et al. 2003) that demonstrated reduced numbers of astrocytes and oligodendrocytes and an increased number of cells moving down the neural lineage in *Sox9*-ablated embryos.

A third paper looked at the function of the endogenous microRNA-124 in the central nervous system which represses SOX9 expression (Cheng, Pastrana et al. 2009). Micro RNAs (miRNAs) are small non-coding RNAs that act as post transcriptional modulators acting on messenger RNA (mRNA) molecules often inhibiting their translation to proteins. This paper shows that *Sox9* is a direct target of miRNA-124 which inhibits the translation of *Sox9* mRNA into SOX9 protein. miRNA-124 is not expressed in neural stem cells or astrocytes but it is expressed in neuroblasts and neurons. The expression of miRNA-124 in neuroblasts would be predicted to reduce SOX9 levels in these cells and allow for neuronal differentiation, while the absence of miRNA-124 would allow for higher levels of SOX9 in glial progenitors, astrocytes, and oligodendrocytes. In keeping with these predictions over expression of miRNA-124 in neural stem cells led to a two-fold increase in neuron production and a one-third reduction in astrocyte production *in vitro*. *In vivo* over expression of miRNA-124 in

neural precursor cells in the subventricular zone of adult mice led to a 1.3-fold increase in postmitotic neurons and a decrease in the number of dividing neural precursor cells. Blocking miRNA-124 expression in neural stem cells eliminated neuron production in culture, and in neural precursor cells in the subventricular zone it caused a 30% decrease in neuron production and a 1.5-fold increase in the overall number of dividing neural precursor cells. All three of these papers argue in favour of the idea that inhibiting SOX9 expression reduces neural stem cell function and proliferation as well as the formation of glial cells, while promoting neuron production (Figure 2).

Using the *FoxJ1*-Cre-ER;*ROSA*-βGal mice it has been demonstrated that neural stem cells activated by spinal cord injury proliferate and migrate to the injury site (Meletis, Barnabe-Heider et al. 2008). Approximately 15% of the neural stem cells that migrate to the lesion differentiate into astrocytes (express the astrocytic marker GFAP) and 2% differentiate into oligodendrocytes (express the oligodendrocyte marker Olig2). The rest fail to express mature neural markers. However, the clear majority of neural stem cells in the ependymal layer and in the lesion express SOX9. The gliogenic actions of SOX9 in neural stem cells at the lesion may be partly responsible for the astrocytosis at the glial scar that may inhibit regeneration and for the absence of new neuron generation after injury.

Figure 2: The neural stem cell lineage without SOX9 expression. Under reduced SOX9 expression neural stem cells have been shown to decrease their proliferation rates as well as their production of glial or glial precursor cells, and increase their production of neuroblasts or neurons. Image modified from University of California, San Francisco, Division of Neonatology website (2009).
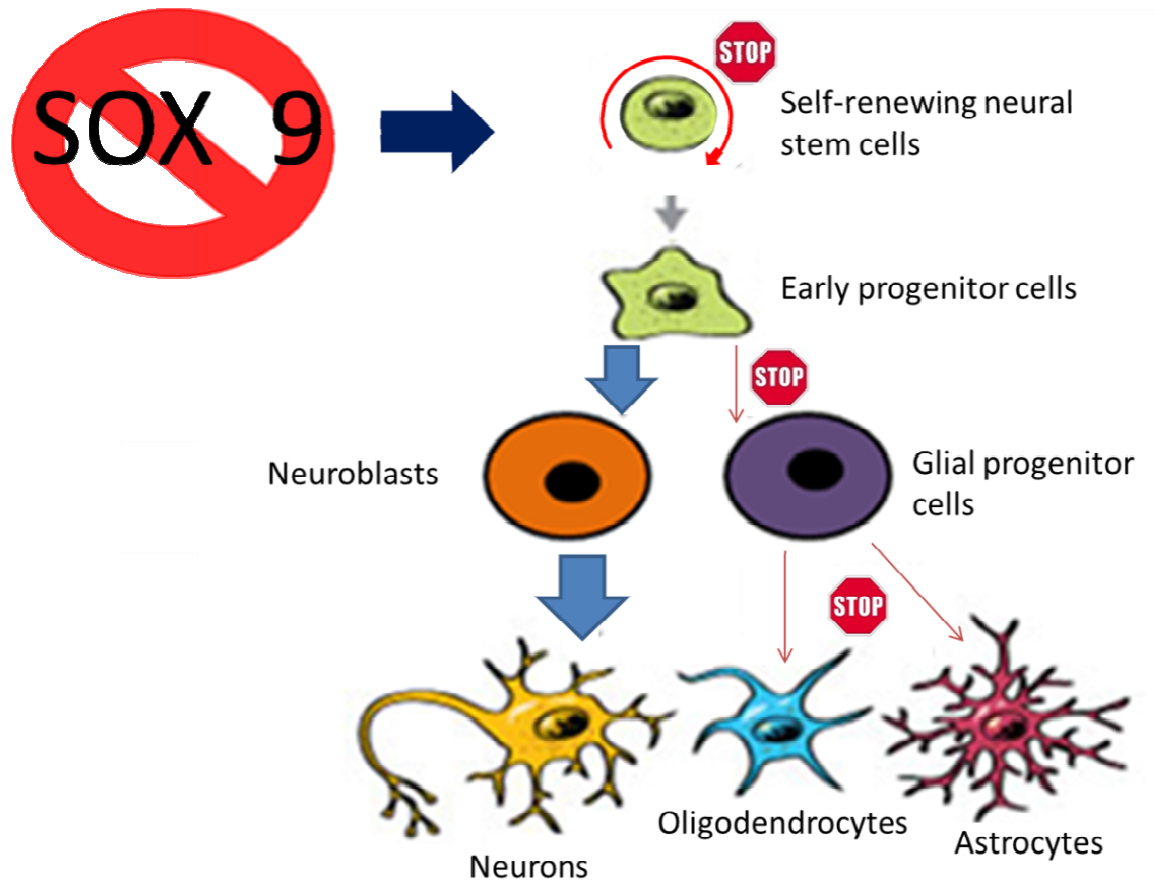
1.7 The role of the transcription factor *Sox9* after spinal cord injury

The Brown laboratory has been studying the role of *Sox9* in recovery from spinal cord injury for several years more for its purported role in controlling matrix gene expression (Gris, Tighe et al. 2007) than for its role in neural stem cell biology. The most prevalent growth inhibiting molecules produced after spinal cord injury are chondroitin sulfate proteoglycans or CSPGs, which are produced by astrocytes and secreted into the extra cellular matrix (Eddleston and Mucke 1993; Fawcett and Asher 1999; Silver and Miller 2004). The large mass of CSPGs produced at the injury site acts as both a physical and chemical barrier to neuron regrowth past the injury preventing recovery of function (Bradbury, Moon et al. 2002). When a growth cone of an extending axon contacts CSPGs they activate protein tyrosine phosphatase (PTP) receptors (Coles, Shen et al. 2011) and signaling pathways that lead to growth cone collapse (McKeon, Schreiber et al. 1991; Davies, Fitch et al. 1997; Zuo, Neubauer et al. 1998). Genetic analysis studies by our lab showed that the transcription factor *Sox9* controls the expression of the genes xylosyltransferase -I and II (XT-I and XT-II) which are responsible for the rate-limiting step in the production of the chondroitin side chains on CSPGs (Gris, Tighe et al. 2007). This led our laboratory to the prediction that *Sox9* ablation would result in lower CSPG levels in the injured cord and hence increased neuroplasticity and improved recovery.

To carry out *Sox9* loss of function studies in a mouse model of spinal cord injury we used two mouse strains. The first strain carries floxed *Sox9* alleles (Akiyama, Chaboissier et al. 2002; Stolt, Lommes et al. 2003) (exons 2 and 3 of *Sox9* surrounded by loxP sites). The second mouse strain is a transgenic line that ubiquitously expresses the Cre recombinase fused to the mutated ligand binding domain of the mouse estrogen

receptor (ER) under the control of the chicken beta actin promoter/enhancer coupled to the Cytomegalovirus (CMV) immediate early enhancer (Hayashi and McMahon 2002). By breeding the *Sox9*^{flox/flox} mice to the CAGGCre-ER mice we generated offspring (*Sox9*^{flox/flox};CAGGCre-ER) in which tamoxifen administration, followed by spinal cord injury allowed us to examine the molecular, cellular, and neurological responses to spinal cord injury in the presence of greatly reduced expression levels of SOX9 in all cell types. Using a 70kdyne contusion spinal cord injury we were able to show that the *Sox9* knockout mice have improved functional recovery of their hindlimbs as measured by the basso mouse scale (BMS) which scores ankle movement and walking ability, as well as increased locomotion using rodent activity boxes compared to normal control mice (McKillop, Dragan et al. 2013). The improved locomotor recovery was correlated with reduced levels of CSPGs at the lesion site as expected.

Why do the *Sox9* conditional knockout mice demonstrate improved locomotor recovery after spinal cord injury? In these conditional knockout mice, tamoxifen administration results in *Sox9* ablation in all cell types. Thus the improved locomotor recovery could be due to effects in astrocytes or neural stem cells (the two major cell types in the spinal cord that express SOX9 (Meletis, Barnabe-Heider et al. 2008). The improved outcomes in this line of mice could possibly be due to the reduced expression of CSPGs at the lesion that may permit greater neuroplasticity, but it could also be due to changes in neural stem cell behaviour. We have argued above that *Sox9* ablation in neural stem cells will generate an increased number of neurons and neuroblasts and a decreased number of astrocytes after spinal cord injury. Oligodendrocytes should also be influenced although this effect should be minimal since the majority of oligodendrocytes produced

after injury are from oligodendrocyte precursors already present in the spinal cord and only a small number come from neural stem cells (Barnabe-Heider and Frisen 2008; Meletis, Barnabe-Heider et al. 2008). If astrocytes differentiated after spinal cord injury contribute to CSPG production, or if neuroblasts are able to generate new neurons or produce growth factors that support neuronal survival (Llado, Haenggeli et al. 2004; Behrstock, Ebert et al. 2006; Madhavan, Ourednik et al. 2008) then the effect of *Sox9* ablation on neural stem cell behaviour may explain the improved recovery of *Sox9* knockout mice after spinal cord injury.

1.8 Background summary

After a spinal cord injury there is very little recovery of function due to the inhibitory environment of the injured spinal cord that blocks axonal growth and neurogenesis. Neural stem cells appear to contribute to these problems by failing to produce new neurons and instead primarily generating astrocytes. Ubiquitous, conditional *Sox9* ablation leads to improved locomotor recovery after spinal cord injury. This thesis seeks to answer several questions: Does *Sox9* ablation alter neural stem cell levels in the injured spinal cord? Does *Sox9* ablation alter neural stem cell differentiation in the injured spinal cord? Does *Sox9* ablation in neural stem cells account for the reported improved recovery in the ubiquitous *Sox9* knockout mice?

1.9 Hypothesis

I hypothesize that conditional ablation of *Sox9* leads to: 1) a decrease in neural stem cells and their progeny at the injury site after spinal cord injury, and 2) a proportional decrease in astrocyte and oligodendrocyte production accompanied by a proportional increase in neuroblasts and neuron production from neural stem cells activated by injury. I further hypothesize that *Sox9* ablation in neural stem cells alone will be sufficient to promote recovery of locomotor function after spinal cord injury.

1.10 Objectives

The purpose of this study is to determine the influence of the transcription factor *Sox9* on the behaviour of neural stem cells after a contusion spinal cord injury. The objectives of the study are to determine:

1. If the levels of neural stem cells and their progeny in the injured spinal cord are reduced by *Sox9* ablation.

2. If the proportion of neural stem cells that differentiate into astrocytes, and oligodendrocytes is reduced in conditional *Sox9* knockout mice after spinal cord injury.

3. If the proportion of neural stem cells that differentiate into neuroblasts and neurons is increased in conditional *Sox9* knockout mice after spinal cord injury.

4. If neural stem cell-specific *Sox9* ablation is sufficient to cause significant improvements in locomotor recovery.

1.11 Overview of experimental plan

Objectives 1-3 will be evaluated using two complementary approaches. The first is to label neural stem cells in the *Sox9*$^{flox/flox}$;CAGGCre-ER mice (in which tamoxifen administration results in ubiquitous *Sox9* ablation as shown in figure 3) with BrdU. In this strain of mice BrdU administration before injury labels a small percentage of the quiescent neural stem cells in the ependymal layer of the spinal cord that can then be followed using anti-BrdU antibodies and immunohistochemistry. This strain of mice has the advantage that we already know that they demonstrate improved recovery after spinal cord injury, but has the disadvantages that only a small percentage of neural stem cells can be followed with this method, BrdU will also label other dividing cells, and that *Sox9* ablation is not stem cell-specific. The second approach makes use of the *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice in which tamoxifen administration restricts *Sox9* ablation and YFP expression (Figure 3) to all neural stem cells and their progeny. This unique strain of mice has the advantage that after tamoxifen administration nearly 100% of neural stem cells can be tracked by their expression of YFP. It also has the advantage of enabling neural stem cell-specific ablation of *Sox9*.

To determine the effect of ubiquitous *Sox9* ablation on neural stem cell levels in the injured spinal cord the number of BrdU-labelled cells in tamoxifen treated *Sox9*$^{flox/flox}$;CAGGCre-ER mice will be compared to the number in tamoxifen treated

$Sox9^{flox/flox}$ mice. To determine the effect of neural stem cell-specific $Sox9$ ablation on neural stem cell levels in the injured spinal cord the number of YFP-expressing cells in tamoxifen treated $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice will be compared to the number in tamoxifen treated $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice. Neural stem cell differentiation patterns will be assessed using immunohistochemical double labelling techniques for either BrdU (in the ubiquitous *Sox9* knockouts) or YFP (in the neural stem cell-specific *Sox9* knockouts) and the cell-specific markers GFAP, APC, DCX and NeuN to delineate astrocytes, oligodendrocytes, neuroblasts and neurons, respectively. To assess functional recovery from spinal cord injury in the neural stem cell-specific *Sox9* knockout mice I will use an open field test for hind limb function and rodent activity boxes to evaluate overall mobility (McKillop, Dragan et al. 2013). These studies will demonstrate the role of *Sox9* in neural stem cell behaviour in the injured mouse spinal cord and determine whether *Sox9* ablation in neural stem cells alone is sufficient to improve locomotor recovery in mice after spinal cord injury.

Figure 3: Representations of the Cre-loxP strategy to knockout the *Sox9* gene and activate

YFP expression. A) In *Sox9* knockout mice, tamoxifen administration allows the Cre-ER

protein to move into the nucleus of the cell (being released from heat-shock protein 90),

and excise exons 2 and 3 of the *Sox9* gene preventing its expression. B) Without

tamoxifen administration, the YFP gene is transcribed into mRNA but a STOP codon

prevents its translation into protein. Tamoxifen administration allows Cre-ER to move

into the nucleus and remove the DNA for this STOP codon leading to YFP protein

expression.

A

Cellular Mechanism for Conditional *Sox9* Knockout:
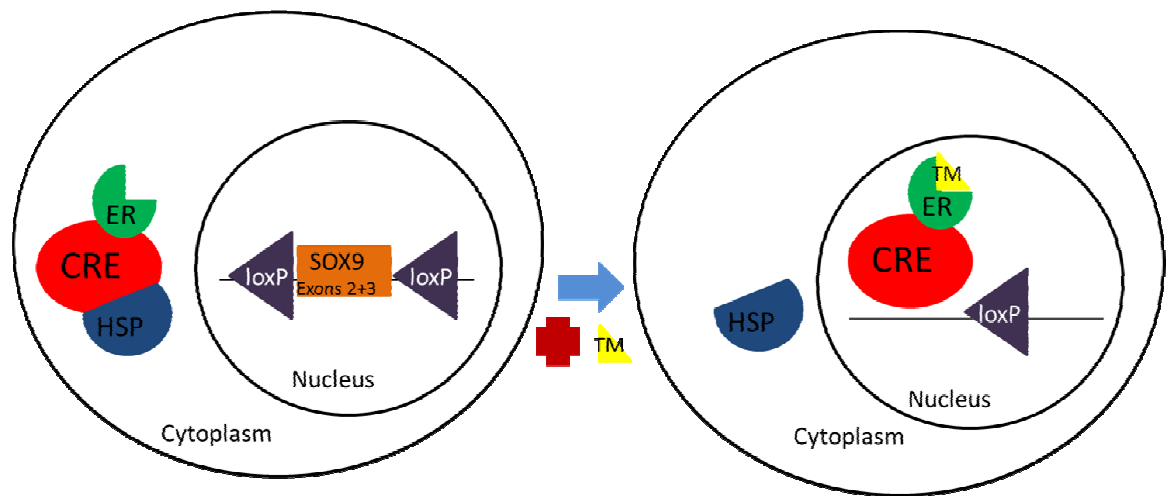
B

Cellular Mechanism for Activation of *YFP* Expression:
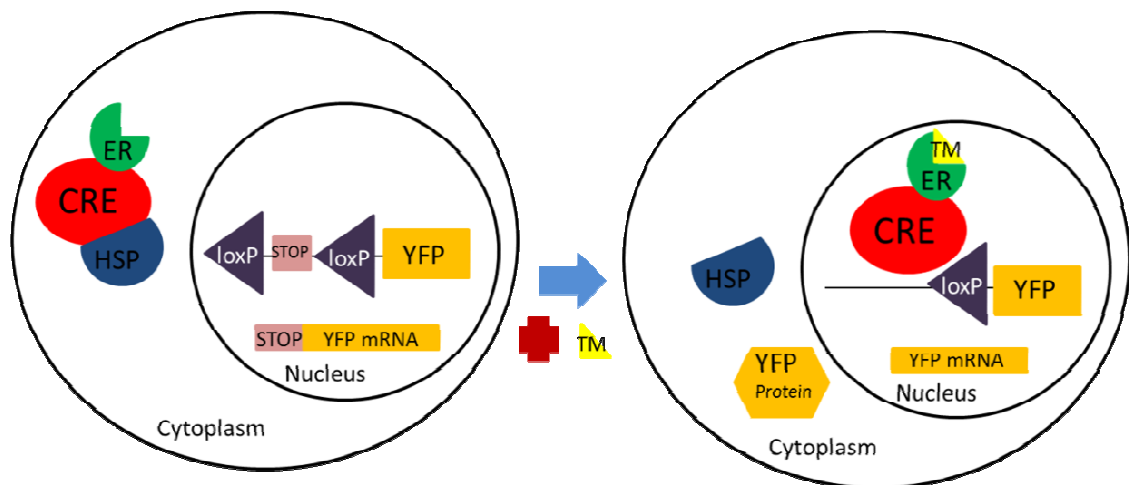
Chapter 2:

Materials and Methods:

2.1 Animals:

2.1.1 *Sox9*^{flox/flox};Cre-ER and *Sox9*^{flox/flox} mice:

To evaluate the absence of SOX9 expression on neural stem cells after spinal cord injury a tamoxifen-inducible conditional *Sox9* knock-out strategy was used. We bred a mouse strain that carries floxed *Sox9* (exons 2 and 3 of *Sox9* surrounded by loxP sites) alleles (Akiyama, Chaboissier et al. 2002) (*Sox9*$^{flox/flox}$) 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) (Hayashi and McMahon 2002) (Jackson Laboratories, Bar Harbor, ME). Tamoxifen administration to *Sox9*$^{flox/flox}$;CAGGCre-ER (*Sox9*$^{flox/flox}$;Cre) mice served as *Sox9* knock-out animals and their tamoxifen treated *Sox9*$^{flox/flox}$ littermates served as control animals. Mice were genotyped by PCR analysis using the following primers:

*Sox9*flox allele: 5'-ACACAGCATAGGCTACCTG-3' and

5'-TGGTAATGAGTCATACACAGTAC-3'.

*Sox9*wildtype allele: 5'-GGGGCTTGTCTCCTTCAGAG-3'

and 5'-TGGTAATGAGTCATACACAGTAC-3'.

Cre+ allele: 5'-CAATTTACTGACCGTACAC-3' and

5'-AGCTGGCCCAAATGTTGCTG-3'.

2.1.2 *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice:

To evaluate the effect of neural stem cell specific *Sox9* ablation after spinal cord injury tamoxifen-inducible, *FoxJ1*-Cre-ER;*ROSA-YFP* mice (Figure 3) (Meletis, Barnabe-Heider et al. 2008) were mated to the *Sox9*^{flox/flox} mice to generate tamoxifen inducible neural stem cell-specific *Sox9* knockout mice (*Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP*) and control litter mates (*Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP*) (Figures 4 and 5). The offspring of this cross were heterozygous for all three of the *FoxJ1*-Cre-ER, *YFP*, and floxed *Sox9* genes. These heterozygous mice were then bred to each other to obtain the desired knockout (*Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP*) and control (*Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP*) genotypes. Tamoxifen administration to the *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* mice results in YFP expression and *Sox9* ablation in neural stem cells and their progeny for the rest of the animal's life. Tamoxifen administration to the *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* littermates also results in YFP expression in neural stem cells and their progeny for the rest of the animal's life. Animals were genotyped by PCR analysis using the three primers mentioned in the previous section for *Sox9*flox allele, *Sox9*wildtype allele, and Cre+ allele, as well as: *YFP*+ allele:

5'-AAGTTCATCTGCACCACCG-3' and

5'-TCCTTGAAGAAGATGGTGCG.

Figure 4: Breeding plan to generate neural stem cell-specific *Sox9* knockout mice. A cross between *FoxJ1*-Cre-ER;*ROSA-YFP* mice and *Sox9*^{flox/flox} mice produces mice heterozygous for the *FoxJ1*-Cre-ER, *YFP*, and floxed *Sox9* genes. These heterozygous mice were bred to each other producing offspring with the potential for many different genotypes, four of which can be used as neural stem cell specific *Sox9* knockout mice. The total probability of a mouse being a neural stem cell specific knockout from this cross is nine out of sixty four or approximately 14%. The probability of a mouse having the proper genotype to be used as a littermate control mouse is the same as the knockout mice, or approximately 14% (not shown).

FoxJ1-CreER   ROSA-YFP   SOX9^{wild}
FoxJ1   ROSA-YFP   SOX9^{wild}

FoxJ1   ROSA-wt   SOX9^{flox}
FoxJ1   ROSA-wt   SOX9^{flox}

50%

50%

FoxJ1   ROSA-YFP   SOX9^{wild}
FoxJ1   ROSA-wt   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{wild}
FoxJ1   ROSA-wt   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{wild}
FoxJ1   ROSA-wt   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{flox} 2/64
FoxJ1   ROSA-YFP   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{flox} 1/64
FoxJ1-CreER   ROSA-YFP   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{flox} 4/64
FoxJ1   ROSA-wt   SOX9^{flox}

FoxJ1-CreER   ROSA-YFP   SOX9^{flox} 2/64
FoxJ1-CreER   ROSA-wt   SOX9^{flox}

*Total Probability of Producing Knockout Mice = 9/64

Figure 5: Electrophoresis gels showing the genotypes of the mice used in the *ROSA-YFP*

reporter gene experiment. Every mouse was genotyped for four genes to determine which

mice were neural stem cell-specific *Sox9* knockouts, controls, or neither. These gels show

a sample of 24 mice. In each gel the presence of a gene is demonstrated by a white band.

The gene interleukin-6 that every mouse possesses is used as a control for each gel.

A,B,C,D) The gels show the results of the polymerase chain reaction for the presence of

the following genes: A) Cre-ER, B) floxed *Sox9*, C) wild-type *Sox9*, and D)*YFP*. For the

YFP gel only those mice that were previously found to be Cre-ER+ were genotyped. For

a mouse to be a neural stem cell-specific *Sox9* knockout it would need to be positive for

the Cre-ER, floxed *Sox9*, and *YFP* genes, and be negative for the wild-type *Sox9* gene.

Out of the 24 mice shown here only 4 met these criteria to be knockouts. They were mice

numbers 1, 2, 6, and 20. The controls were mice numbers 3, 12, and 18.

A Cre Genotype
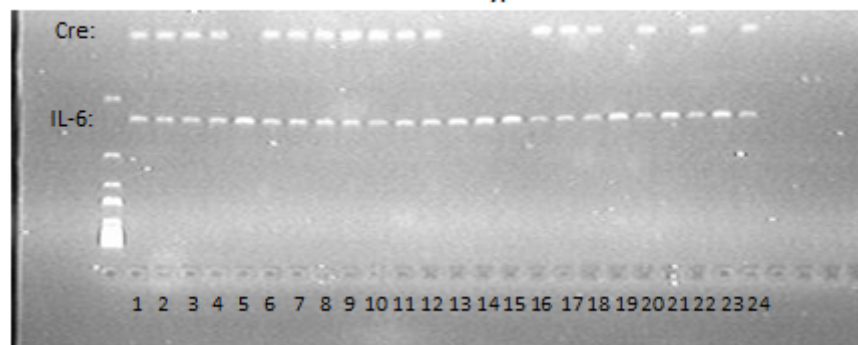
B Sox9 Floxed Genotype
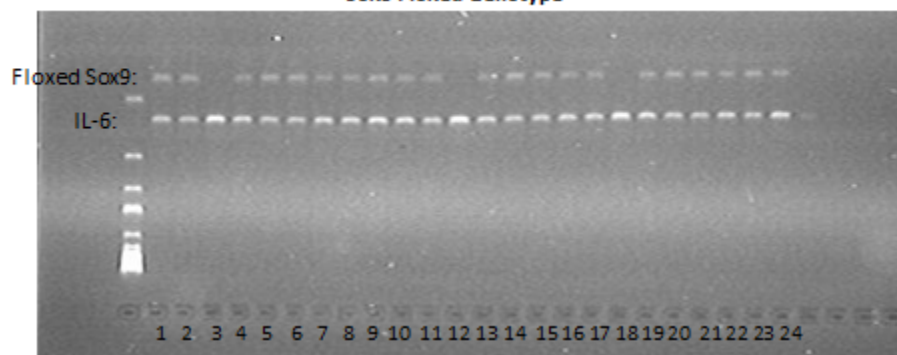
C Sox9 Wildtype Genotype
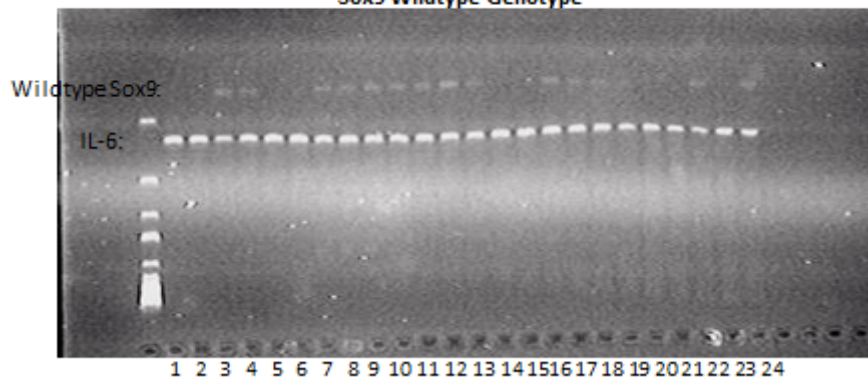
D YFP Genotype
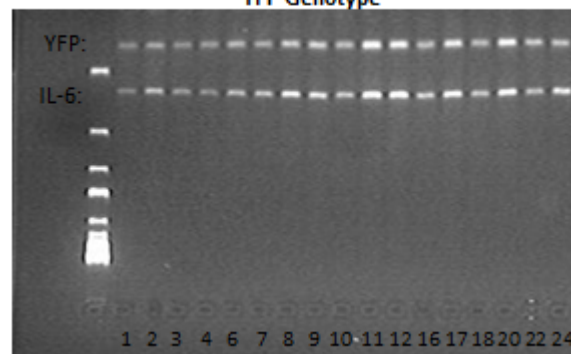
2.2 BrdU Administration:

To label neural stem cells in the spinal cords of the $Sox9^{flox/flox}$;Cre-ER and

$Sox9^{flox/flox}$ mice, we administered 5-bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich, St.

Louis, MO, product # B5002) to the mice in their drinking water at 1mg/mL for 3 weeks.

The water was changed twice a week and kept in foil-wrapped bottles. The cages were

changed and cleaned in a fume hood and all bedding was incinerated. Some of the BrdU

administered mice were not given spinal cord injury surgeries to provide BrdU base-line

labelling levels before injury. To compare the BrdU and YFP neural stem cell labelling

efficiencies BrdU was also administered to 4 $SOX9^{wild/wild}$;$FoxJ1$-Cre-ER;$ROSA$-YFP

control mice, and these mice were not given spinal cord injuries.

2.3 Tamoxifen Administration:

Tamoxifen (Sigma Aldrich, St. Louis, MO, product # T5648) was administered at

3 mg/20 g mouse by oral gavage to all $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ littermates once

a day for 7 days after the last day of BrdU treatment. Tamoxifen was administered to all

$Sox9^{flox/flox}$;$FoxJ1$-Cre-ER;$ROSA$-YFP and $SOX9^{wild/wild}$;$FoxJ1$-Cre-ER;$ROSA$-YFP mice

in the same way, but at the beginning of their experiment. Following the last day of

tamoxifen oral gavage, the animals were housed for 7 days without any treatment to

allow time for Cre-mediated recombination and tamoxifen clearance before subsequent

spinal cord injury.

2.4 Surgeries:

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, 12 female *Sox9*$^{flox/flox}$;Cre-ER and 12 female *Sox9*$^{flox/flox}$ mice, as well as 15 female and 15 male *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice and 15 female and 15 male *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* 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 T8 and T10 with forceps. The T9 spinal segment was injured by a 50 kdyne contusion in the *Sox9*$^{flox/flox}$;Cre-ER and *Sox9*$^{flox/flox}$ mice, and a 70 kdyne contusion in the *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice, each delivered with a 1 s dwell time by a computer-controlled Impactor (displacement range: 500–1000 lM) (Infinite Horizons Impactor, Precision Systems and Instrumentation, Fairfax, VA). Following injury 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 after injury and then twice a day for the next 3 days post spinal cord injury. Bladders were manually emptied twice daily for the duration of the experiment. Three *Sox9*$^{flox/flox}$;Cre-ER and three *Sox9*$^{flox/flox}$ mice were not given spinal cord injury and were used as uninjured controls for BrdU labelling efficiency. *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice were kept only if their surgeries produced injuries with displacements between (500-1000µM), with forces within 10% of the desired 70kdynes

(63-77kdynes) and their BMS scores were ≤0.5 the day after injury. These rigorous criteria for inclusion in the study resulted in a large number of animals being excluded from analyses so that at the end of the experiment there were 6 female and 4 male $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice and 7 female and 3 male $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice.

2.5 Behaviour:

Locomotor recovery of the $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice was assessed by two blinded observers using the Basso Mouse Scale (BMS) open field locomotor score (Basso, Beattie et al. 1995). The day following spinal cord injury, 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 neural stem cell-specific *Sox9* conditional knockouts and 2 controls). Animals were evaluated once a week for 11 weeks after spinal cord injury. Left and right hind limb scores were averaged to generate a composite score. In addition, overall locomotion was evaluated after tamoxifen treatment but prior to injury, and at 4, 8, and 11 weeks post spinal cord injury using rodent activity boxes (Accuscan Instruments Inc, Columbus, OH). Activity boxes record the distance traveled by detecting breaks in a series of infrared light beams. The total distance traveled by each mouse was measured over a 2-h period at night (during their normal awake circadian cycle).

2.6 Perfusions:

Animals were deeply anesthetized with 100mg/kg ketamine: 5mg/kg xylazine and cardiac perfusions were performed using saline solution followed by 4% paraformaldehyde (4% PFA in 0.1 M phosphate buffer at pH 7.4). The $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ mice were perfused two weeks after spinal cord injury (experiment timeline shown in Figure 6), and due to mortality and the specific requirments for the displacement and force measurements during surgery, there were 6 female $Sox9^{flox/flox}$;Cre-ER and 9 female $Sox9^{flox/flox}$ mice at the end of the study. The $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice were perfused after behavioural testing was complete 11 weeks after spinal cord injury (experiment timeline shown in Figure 6). Spinal cords were dissected and postfixed for 1 h in 4% PFA and then cryoprotected in 20% sucrose at 4°C overnight. Spinal cords were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A. Inc., Torrance, CA), frozen over dry ice, and stored at -80°C overnight. 4.8mm of the frozen cords from $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ mice, and 8mm of the frozen cords from $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice, were then cross-sectioned (with the epicenter of the injury used as the midpoint) at 16 µm using a cryostat, and serially thaw-mounted on SuperfrostTM glass slides (Fisher Scientific Company, Ottawa, Canada). Collected sections alternated on 10 slides with 10 sections per slide so each section per slide is separated by 160µm. Alternating sections on 10 different slides at a time prevents double counting of cells since only one slide from each series of 10 is used to quantify each type of cell marker.

Figure 6: Experimental timelines for both experiments discussed in this thesis.

A) Timeline for the experiment using BrdU to label neural stem cells in the ubiquitous

Sox9 knockout mice (*Sox9*^{flox/flox};Cre-ER) and their littermate controls (*Sox9*^{flox/flox}).

B) Timeline for the experiment using YFP to label neural stem cells in the neural stem

cell-specific *Sox9* knockout mice (*Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP*) and their

littermate controls (*Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP*).

A

Ubiquitous *Sox9* Knockout Mice Experiment Timeline

50 kdyne, T9
contusion SCI

Week: 0   1   2   3   4   5   6   7

BrdU treatment
(1mg/mL in
drinking water)

Tamoxifen
treatment

Recombination
(tamoxifen
clearance)

Perfusions for
protein analysis by
immunohistochemistry

B

Neural Stem Cell-Specific *Sox9* Knockout Mice Experiment Timeline

70 kdyn, T9
contusion SCI

Behaviour Testing (BMS
and rodent activity boxes)

Week: 0   1   2   3   4   5   6   7   8   9   10   11   12   13

Tamoxifen
treatment

Recombination
(tamoxifen
clearance)

Perfusions for protein
analysis by
immunohistochemistry
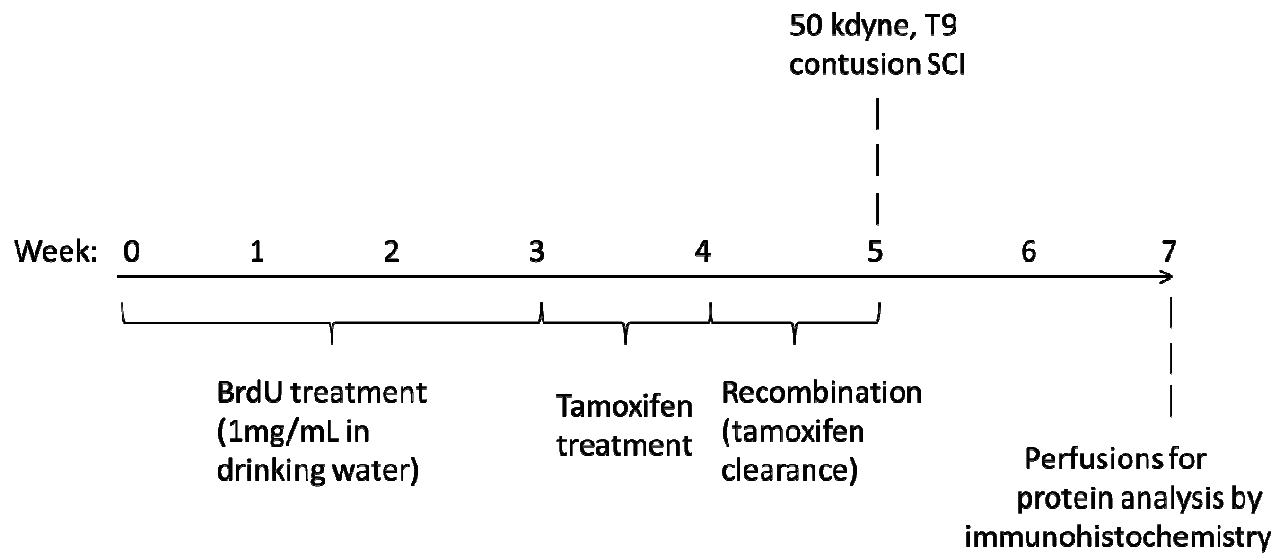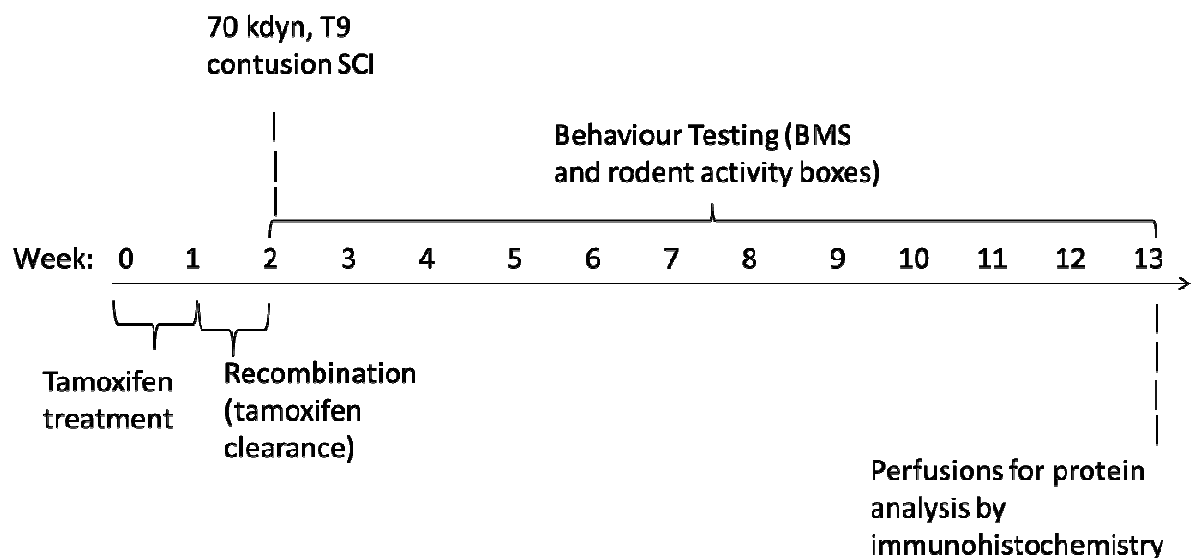
2.7 Immunohistochemistry:

Sections from $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ mice were incubated with blocking solution (5% goat serum in PBS, with 0.1% Triton X-100) for 2 h at room temperature, then incubated with primary antibodies diluted in blocking solution at 4°C overnight. The following primary antibodies were used: mouse anti-GFAP (1:200, Millipore, product # MAB360), mouse anti-DCX (1:200, BD Biosciences, product #611706), rabbit anti-Sox9 (1:300, Millipore, product # AB5535), mouse anti-NeuN (1:200, Millipore, product # MAB377). After washing 3 times for 10 minutes in PBS immunofluorescent labelling was performed using the following secondary anti-bodies: Alexa Floura 594 donkey anti-mouse IgG (1:500, Invitrogen, product # A21203) and Alexa Floura 594 donkey anti-rabbit IgG (1:500, Invitrogen, product # A21207) for one hour at room temperature. After washing 3 times for 30 minutes the sections were incubated in 4% PFA for 30 minutes at room temperature to fix the sections before acid treatment. After washing 3 times for 10 minutes in PBS the sections were incubated in 1 M HCl at 37°C for 45 minutes to break down the DNA to enable BrdU labelling. The sections were then neutralized by incubation in TRIS buffer (pH 8) for 15 minutes. After washing 2 times in PBS for 5 minutes sections were re-incubated in blocking solution for 2 h at room temperature, then incubated with the primary antibody rat anti-BrdU (1:200, AbD Serotec, product # OBT0030G) diluted in blocking solution at 4°C overnight. After washing 3 times in PBS for 10 minutes immunofluorescent labelling of BrdU was performed using the secondary antibody Alexa Floura 488 donkey anti-rat IgG (1:500, Invitrogen, product # A21208) for one hour at room temperature. After washing 3 times in PBS for 1 hour sections were coverslipped with ProLong Gold Anti-Fade mounting

medium (Invitrogen, Carlsbad, CA). The same protocol was used to stain sections for YFP and BrdU from the 4 *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* control mice, but using the primary antibody rabbit anti-GFP (1:300, Life Technologies, product #A11122) and the secondary antibody Alexa Floura 488 goat anti-rabbit IgG (1:500, Life Technologies, product #A11034) to stain for YFP, before staining for BrdU.

Sections from *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice were incubated with blocking solution (5% carnation bovine skim milk in PBS) for 2 h at room temperature, and then incubated with primary antibodies diluted in blocking solution at 4°C for two nights. The following primary antibodies were used: mouse anti-GFAP (1:500, Millipore, product # MAB360), mouse anti-DCX (1:500, BD Biosciences, product #611706), rabbit anti-Sox9 (1:300, Millipore, product # AB5535), mouse anti-NeuN (1:200, Millipore, product # MAB377), mouse anti-APC (1:100, CalBioChem, product # OP80), rabbit anti-GFP (1:2000, Life Technologies, product #A11122), chicken anti-GFP (1:2000, Abcam, product #ab13970), mouse anti-CS56 IgM (1:300, Sigma Aldrich, product #C8035). In all instances of YFP staining (which is done using anti-GFP antibodies) the rabbit anti-GFP antibody was used except for double staining with SOX9 where chicken anti-GFP was used instead.

After washing 3 times for 10 minutes in PBS, immunofluorescent labelling was performed using the following secondary anti-bodies: Alexa Floura 594 donkey anti-mouse IgG (1:500, Invitrogen, product # A21203), Alexa Floura 594 donkey anti-rabbit IgG (1:500, Invitrogen, product # A21207), Alexa Floura 488 goat anti-rabbit IgG (1:500, Life Technologies, product #A11034), Alexa Floura 488 goat anti-chicken IgG (1:500, Life Technologies, product #A11039), Alexa Floura 594 goat anti-mouse IgM (1:500,

Life Technologies, product #A21044) for one hour at room temperature. After washing 3

times in PBS for at least 30 minutes each time the sections were coverslipped with

ProLong Gold Anti-Fade mounting medium (Invitrogen, Carlsbad, CA).

2.8 Microscopy:

Stained sections were analyzed for cell-specific markers using Image Pro Plus

software (Media Cybernetics, Inc., Bethesda, MD). Every section was analyzed on each

slide and one slide from each series of 10 slides was stained for every double labelling

marker combination that was quantified. For the $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ mice

1.6mm of their spinal cords were analyzed centered on the epicenter of the injury.

Pictures were taken of every BrdU+ cell at 40X magnification and each cell was

individually analyzed to determine if it overlapped with a DAPI+ cell nucleus. Once

confirmed that the DAPI+ nucleus was BrdU+ this cell was analyzed for positive signals

of the other antibody markers used. For the $Sox9^{flox/flox}$;$FoxJ1$-Cre-ER;$ROSA$-YFP and

$Sox9^{wild/wild}$;$FoxJ1$-Cre-ER;$ROSA$-YFP mice 4.8mm of their spinal cords were analyzed

centered on the epicenter of the injury. Pictures were taken at 40X magnification of every

YFP+ cell and it was determined which DAPI+ nuclei the YFP signal was surrounding.

Each DAPI+ nuclei from a cell that was also YFP+ was individually analyzed for positive

signals of the other antibody markers used. YFP+ cells in spinal cord sections with an

intact ependymal layer where no neural stem cell activation was seen were not quantified

and the sections that were >3.2mm rostral from the epicenter of the injury were used as

control sections in uninjured parts of the spinal cord. Several pictures of the stained

sections were taken with a confocal microscope. CS-56 was quantified using the Image Pro Plus software to quantify the total area of immunoreactivity of the antibody.

For the 4 $Sox9^{wild/wild}$;$FoxJ1$-Cre-ER;$ROSA$-YFP control mice that were given BrdU administration, every cell that was BrdU and YFP positive was quantified as a percentage of total YFP+ cells in the ependymal layer of the spinal cords, to compare the labelling efficiencies of each method.

2.9 Statistics:

Mean values are expressed ±SE. BrdU and YFP double-labelling, and YFP proliferation quantifications were subjected to statistical analysis using a Student's t-test. YFP and SOX9 quantification, and the YFP proliferation data split into areas at the epicenter, and rostral and caudal to the lesion, were subjected to a one-way ANOVA. The BMS and the locomotion box behavioural testing results were subjected to a two-way repeated measures ANOVA. Analyses were conducted with GraphPad Prism 4.0 software (Graphpad Software Inc., La Jolla, CA), and significance was accepted at $P < 0.05$.

Chapter 3:

Results:

3.1 BrdU labelling efficiency

While intraperitoneal injections of BrdU in mice is often used to label fast dividing cells, BrdU in drinking water is more useful for labelling slowly dividing cells (Khodosevich, Watanabe et al. 2011). For this reason we chose to administer BrdU to the ubiquitous *Sox9* knockout and control mice in their drinking water for 3 weeks to allow for more cell divisions to take place to increase the labelling percentage of ependymal cells. The BrdU administration was followed by tamoxifen administration to activate Cre-ER. The wild-type (Cre-ER negative) control mice also received tamoxifen. After one week of washout the mice were given a contusion spinal cord injury at the level of the ninth thoracic vertebra (T9) with a force of 50 kilodynes. We waited two weeks after spinal cord injury before perfusing the mice to allow time for the neural stem cells to proliferate and differentiate. As shown in Figure 7, before spinal cord injury BrdU positive cells are found almost exclusively in the ependymal layer of the spinal cord, almost always in pairs since a cell must divide in order to be labelled, and there is no significant difference between the *Sox9* knockout mice and the control mice in the percentage of their ependymal layer that is BrdU+ before injury.

Figure 7: There is no difference in the labelling efficiency of BrdU between *Sox9*

knockout mice and control mice. A,B) BrdU labels ependymal cells (green), which is

where the neural stem cells are located, in pairs (arrows) in an uninjured wild-type mouse

(A) and a *Sox9* knockout mouse (B). Scale bars indicate 50µm. C) Quantification of the

percent of the ependymal layer that was BrdU+ in uninjured mice showed no difference

between *Sox9* knockout mice and control mice as demonstrated by a student's t-test

(p>0.05, N= 3 *Sox9* KO, 3 controls).

A

B
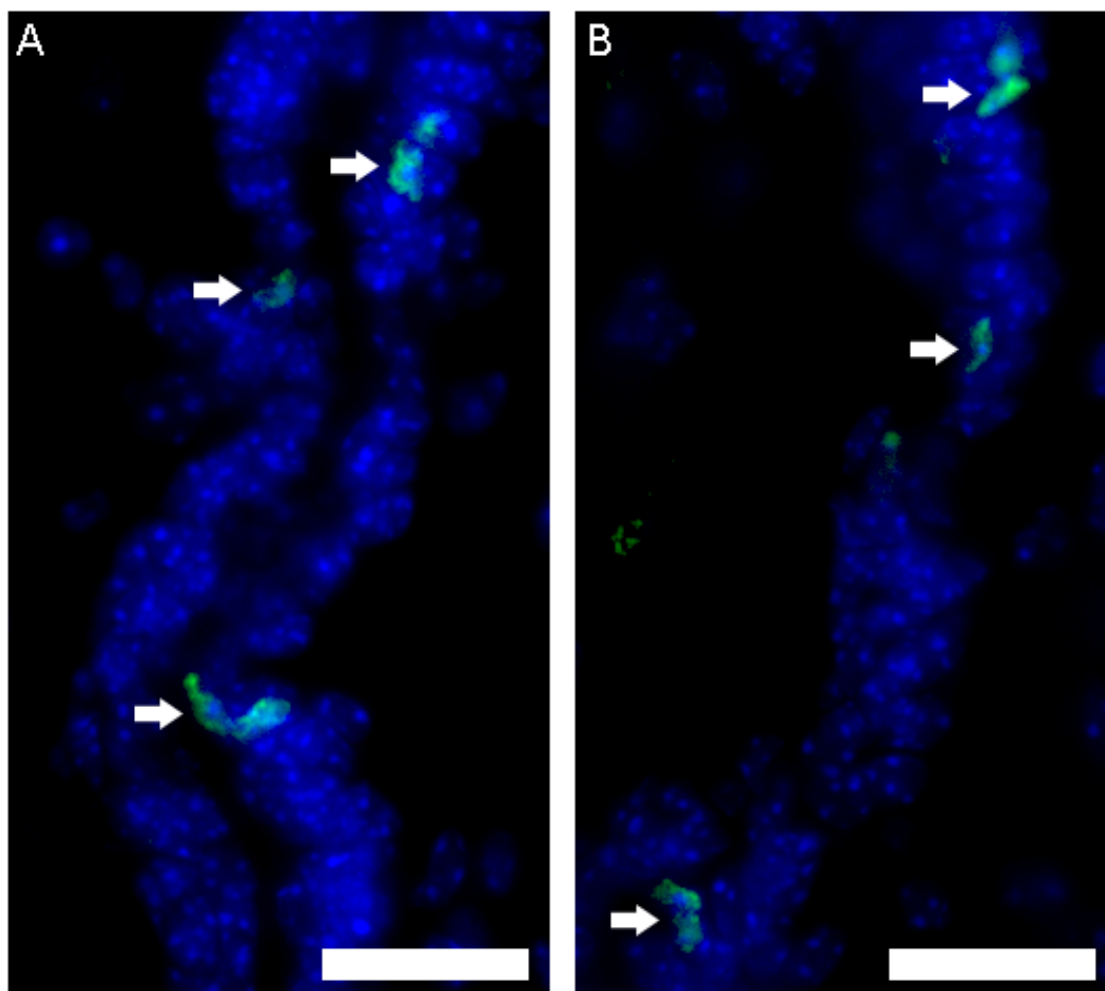
C

BrdU Labelling Efficiency of
NSCs in uninjured Mice
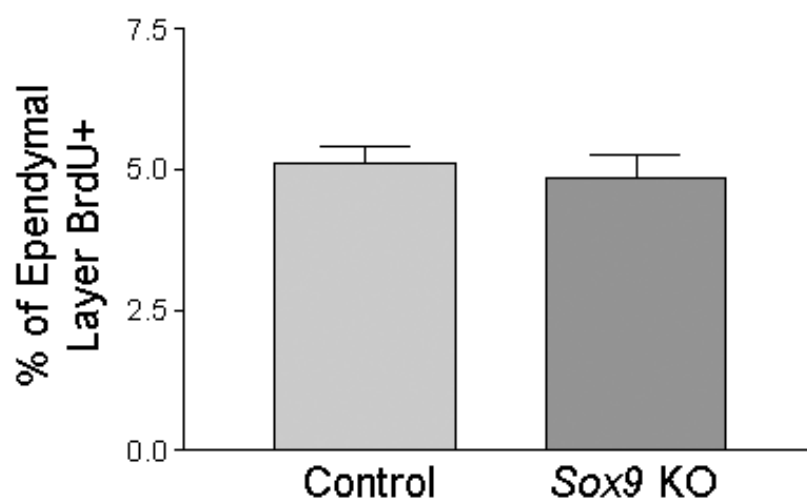
To estimate the percentage of neural stem cells labelled by BrdU we used the same BrdU administration in $Sox9^{wild/wild}$;$FoxJ1$-Cre-ER;$ROSA$-YFP mice. In these mice approximately 90% of neural stem cells express YFP after tamoxifen administration (Meletis, Barnabe-Heider et al. 2008). $FoxJ1$-Cre-ER;$ROSA$-YFP mice were given BrdU in their drinking water for three weeks and then administered tamoxifen to activate YFP expression in the ependymal layer of the spinal cord. After one week to allow for recombination the mice were perfused without being given an injury. The average percent of YFP+ ependymal cells that were also labelled with BrdU was 6.1% (+/- 0.3%) (Figure 8). Therefore, since YFP only labels approximately 90% of neural stem cells in the spinal cord, in our BrdU experiment we only labelled approximately 5 - 6%.

3.2 Investigating neural stem cell behaviour after spinal cord injury in $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ mice using BrdU administration

3.2.1 Tamoxifen administration results in a Cre-mediated recombination frequency of approximately 56% in BrdU positive cells in $Sox9^{flox/flox}$;Cre-ER mice

Sections from tamoxifen-treated $Sox9^{flox/flox}$;Cre-ER and $Sox9^{flox/flox}$ littermates 2 weeks post-spinal cord injury were stained with anti-BrdU antibodies and anti-SOX9 antibodies. In control sections 84.1% of the BrdU-labelled cells also expressed SOX9 whereas only 36.6% of BrdU-labelled cells also expressed SOX9 in sections from $Sox9^{flox/flox}$;Cre-ER mice (Figure 9). This indicates that SOX9 expression in the BrdU positive cells in the spinal cord was reduced by 56% in the $Sox9$ knockout mice.

Figure 8: *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice are 94% more efficient at labelling neural stem cells than BrdU administration. A) BrdU+ cells (red) are found in pairs (arrows) in the ependymal layer of an uninjured *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mouse whose ependymal layer expresses YFP (green). Scale bar indicates 100μm. B) Three weeks of BrdU administration in the water of *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice is only able to label 6.1% of the YFP+ neural stem cells in the ependymal layer of the spinal cord. N= 3 *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice.

A
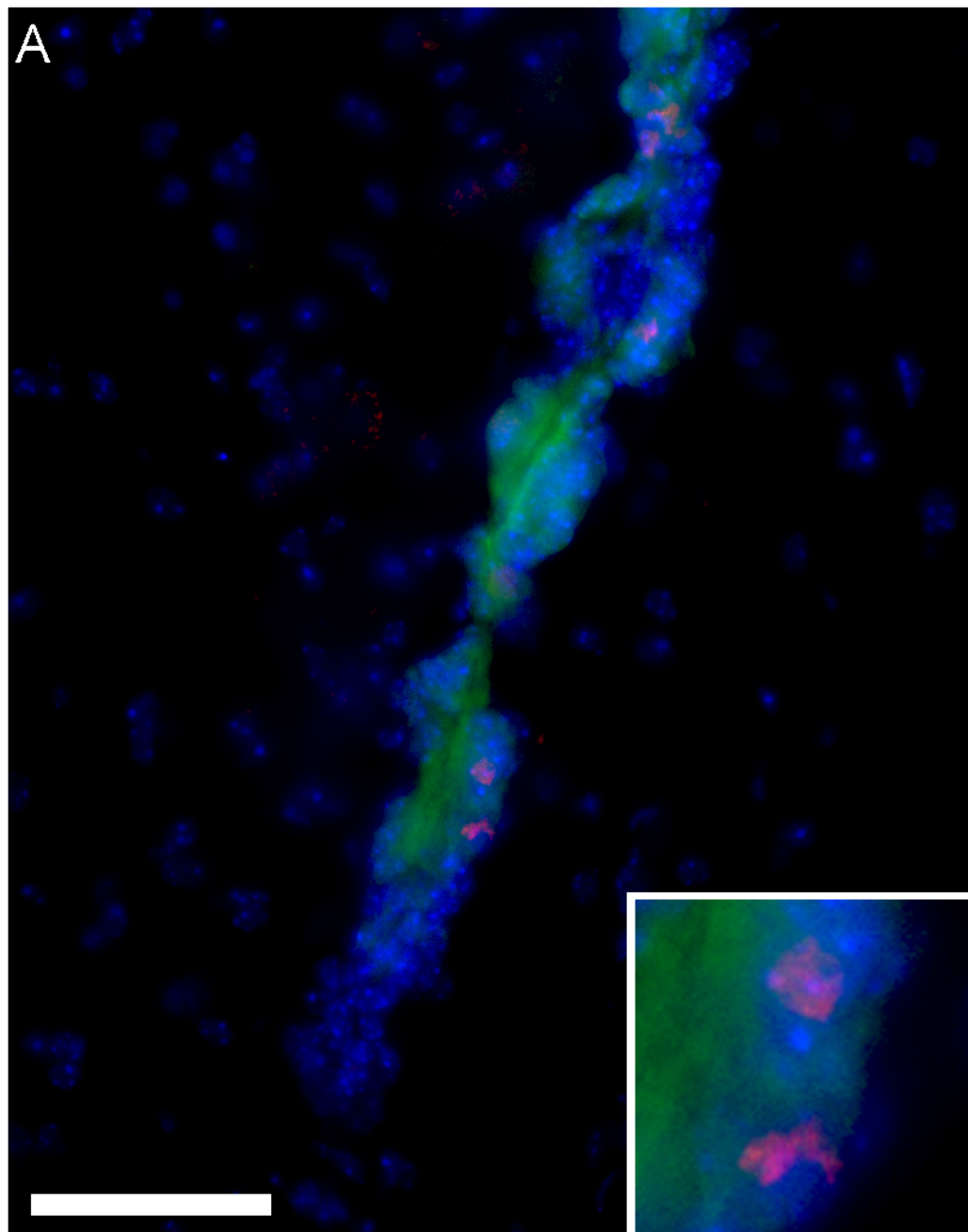
B

YFP is More Efficient at Labelling
NSCs than BrdU

FoxJ1 Mice
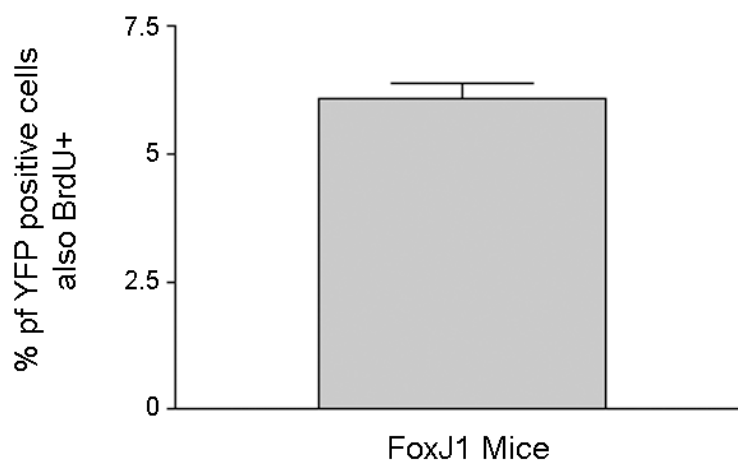
Figure 9: SOX9 expression is reduced by 56% in the BrdU+ cells in the *Sox9* knockout

mice compared to wild-type mice. A) A BrdU+ (green) and SOX9+ (red) cell in a wild-

type mouse after spinal cord injury. B) A BrdU+ and SOX9+ cell is seen along with two

BrdU+ cells that are not SOX9+ in a *Sox9* knockout mouse after injury. Scale bars

indicate 100μm. C) Quantification of the percentage of BrdU+ cells that are also SOX9+

shows a significant reduction in the *Sox9* knockout mice compared to control mice as

demonstrated by a student's t-test (p<0.05, N= 6 *Sox9* KO, 9 controls).

C

Efficiency of *Sox9* KO in NSCs
and Their Progeny
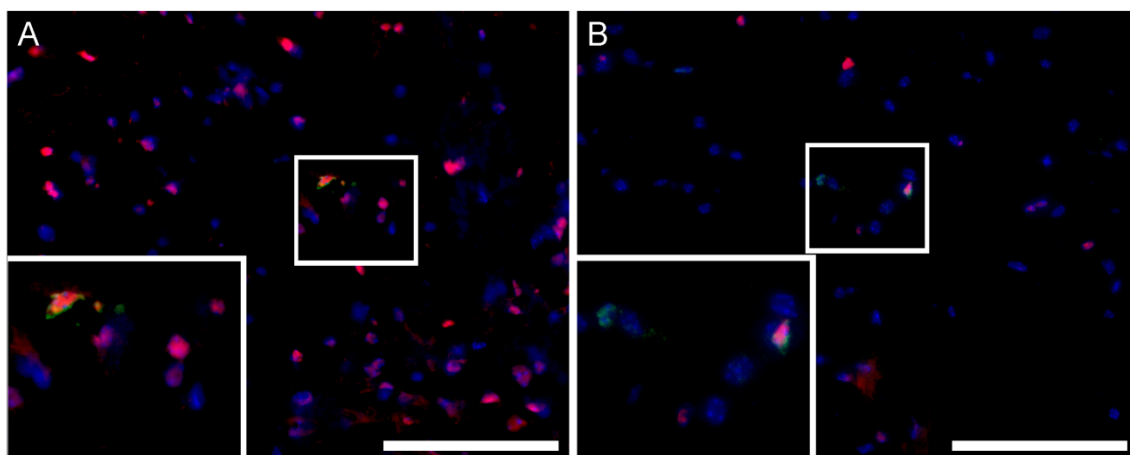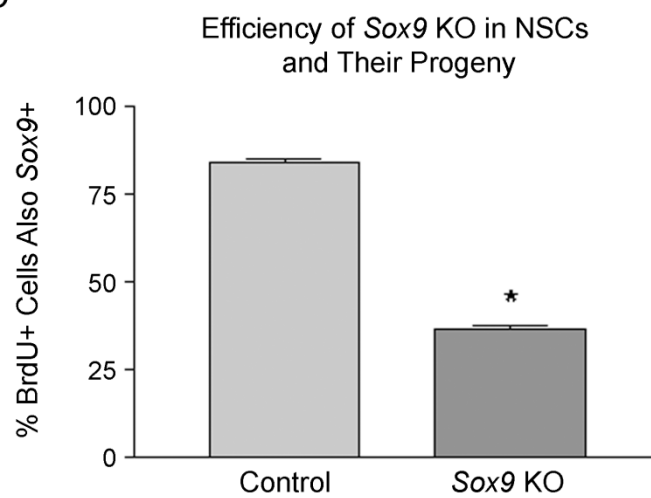
3.2.2 Neural stem cell levels after spinal cord injury are unaffected by ubiquitous *Sox9* ablation

To investigate the effects of ubiquitous *Sox9* ablation on neural stem cell proliferation and survival sections from control and $Sox9^{flox/flox}$;Cre-ER mice at 2 weeks post-spinal cord injury were immunostained for BrdU. The number of BrdU labelled cells in control and $Sox9^{flox/flox}$;Cre-ER sections were not significantly different from one another (Figure 10).

3.2.3 Neural stem cells produce fewer astrocytes after spinal cord injury in ubiquitous *Sox9* conditional knockout mice

We studied the differentiation patterns of the neural stem cells in control mice and ubiquitous *Sox9* knockout mice. The number of new-born astrocytes, neuroblasts and neurons were evaluated 2 weeks post spinal cord injury by counting the number of BrdU+ cells that also expressed GFAP, DCX and NeuN, respectively. This analysis revealed a small but statistically significant reduction in the percentage of BrdU+ cells that express GFAP in the ubiquitous *Sox9* knockout mice (9.6% versus 11.7%) (Figure 11), but failed to reveal any BrdU+/DCX or BrdU+/NeuN expressing cells.

Figure 10: No change was found in neural stem cell proliferation after spinal cord injury between ubiquitous *Sox9* knockout mice and control mice. A,B) Proliferation of BrdU positive cells after spinal cord injury in a control mouse (A) and a ubiquitous *Sox9* knockout mouse (B). Scale bars indicate 100µm. C) No change was found in the total number of BrdU+ cells after spinal cord injury between ubiquitous *Sox9* knockout mice and control mice as demonstrated by a student's t-test (p>0.05). N=6 *Sox9* KO, 9 controls
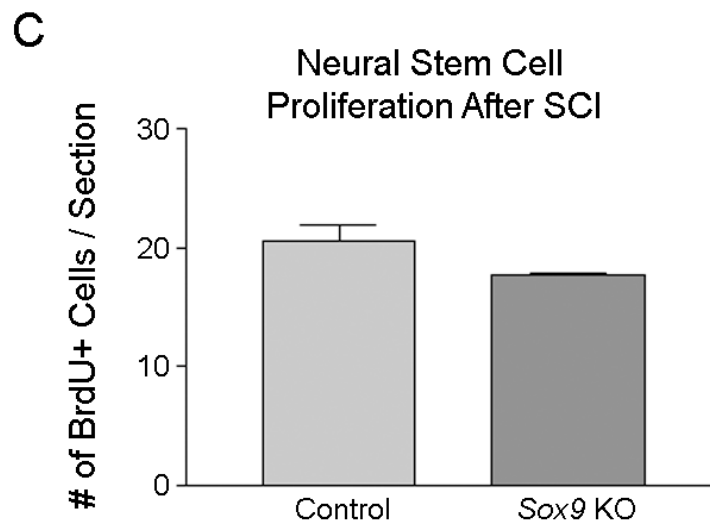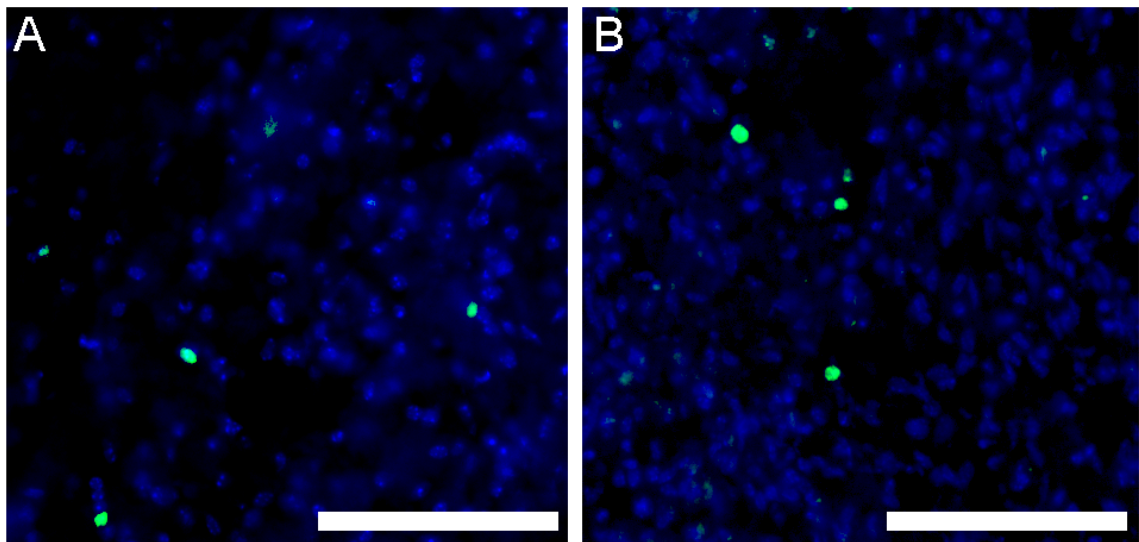
Figure 11: Ubiquitous *Sox9* knockout mice display reduced GFAP and BrdU double labelled cells after spinal cord injury compared to control mice. A, B) A BrdU and GFAP positive cell (indicated by arrows) representing a newly formed astrocyte after spinal cord injury in a control mouse (A) and a ubiquitous *Sox9* knockout mouse (B). Scale bars indicate 50µm. C) *Sox9* knockout mice display reduced GFAP and BrdU positive cells as a percentage of overall BrdU cells as determined by a student's t-test (p<0.05). N= 6 *Sox9* KO, 9 controls.

Neural Stem Cell Derived
Astrocytes After SCI
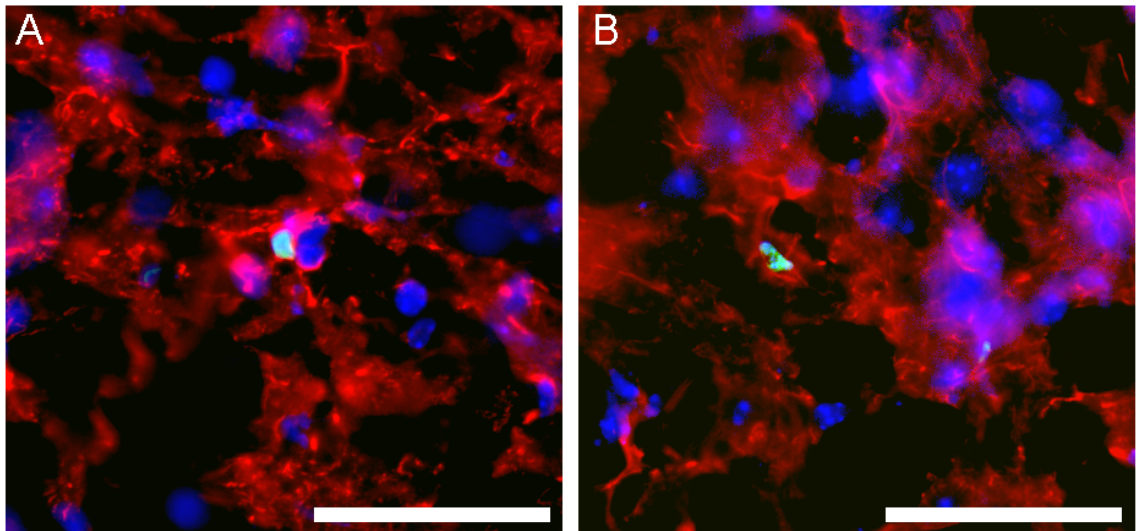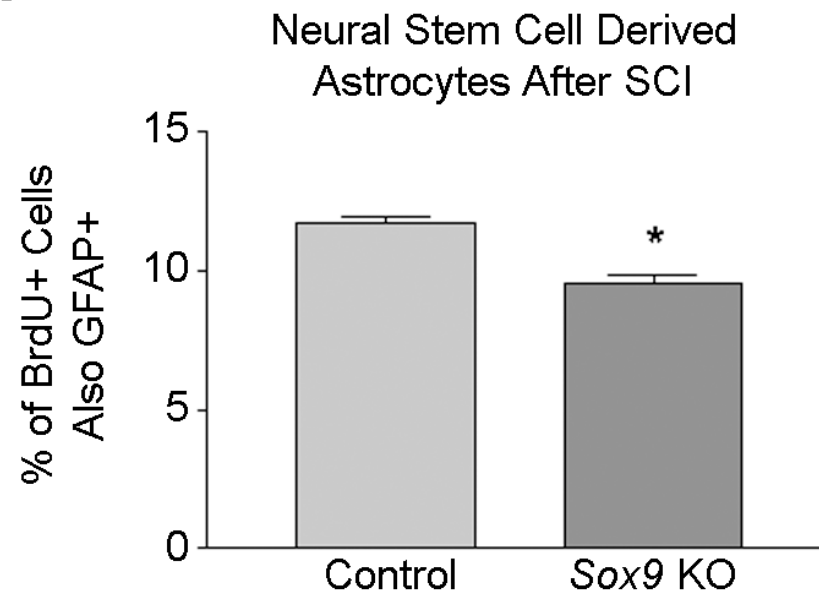
3.3 Investigation of neural stem cell behaviour and neurological functional recovery after spinal cord injury in *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice

3.3.1 Tamoxifen administration results in a Cre-mediated recombination frequency of approximately 65% in neural stem cells in *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* mice

To estimate the frequency with which tamoxifen administration achieved *Sox9* ablation we double immunostained sections from *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice for YFP and SOX9 expression. This analysis revealed that, in the area of the lesion, whereas an average of 66.4% of YFP-expressing cells in control sections expressed SOX9 only 23% of YFP-expressing cells also express SOX9 in the *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* sections (Figure 12). Outside the area of the lesion epicenter (>3.2 mm rostral) the percentage of YFP/SOX9 double labelled cells in *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* mice is only 17.7%, showing SOX9 expression increased after spinal cord injury.

3.3.2 Neural stem cell levels after spinal cord injury are reduced in neural stem cell-specific *Sox9* knockout mice

To evaluate the effect of neural stem cell specific *Sox9* ablation on neural stem cell and neural stem cell progeny levels in the injured spinal cord, sections from tamoxifen-treated *Sox9*^{flox/flox};*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*^{wild/wild};*FoxJ1*-Cre-ER;*ROSA-YFP* mice were immunostained for YFP expression at 11 weeks post-spinal

Figure 12: Neural stem cell-specific *Sox9* knockout mice have reduced SOX9 expression following spinal cord injury in uninjured and injured spinal cord sections. A, B) SOX9 expression (red) in YFP+ ependymal cells (green) in uninjured spinal cord sections in a wild-type mouse (A) and a neural stem cell-specific *Sox9* knockout mouse (B). C, D) SOX9 expression (red) in YFP+ neural stem cells and their progeny (green) 11 weeks after spinal cord injury in a wild-type mouse (C) and a neural stem cell-specific *Sox9* knockout mouse (D). All pictures were taken with a confocal microscope. Scale bars indicate 10µm. E) Quantification of SOX9 expression in YFP+ cells found that SOX9 is significantly reduced in neural stem cell-specific *Sox9* knockout mice compared to control mice both in uninjured and injured spinal cord sections. SOX9 expression in YFP+ cells in neural stem cell-specific *Sox9* knockout mice was also found to be significantly increased in the injured spinal cord sections compared to the uninjured spinal sections. All results were demonstrated by a one-way ANOVA, Bonferroni post-hoc test ($p < 0.05$, N= 9 NSC-*Sox9* KO, 9 controls).

A Control   B NSC-*Sox9* KO

C   D
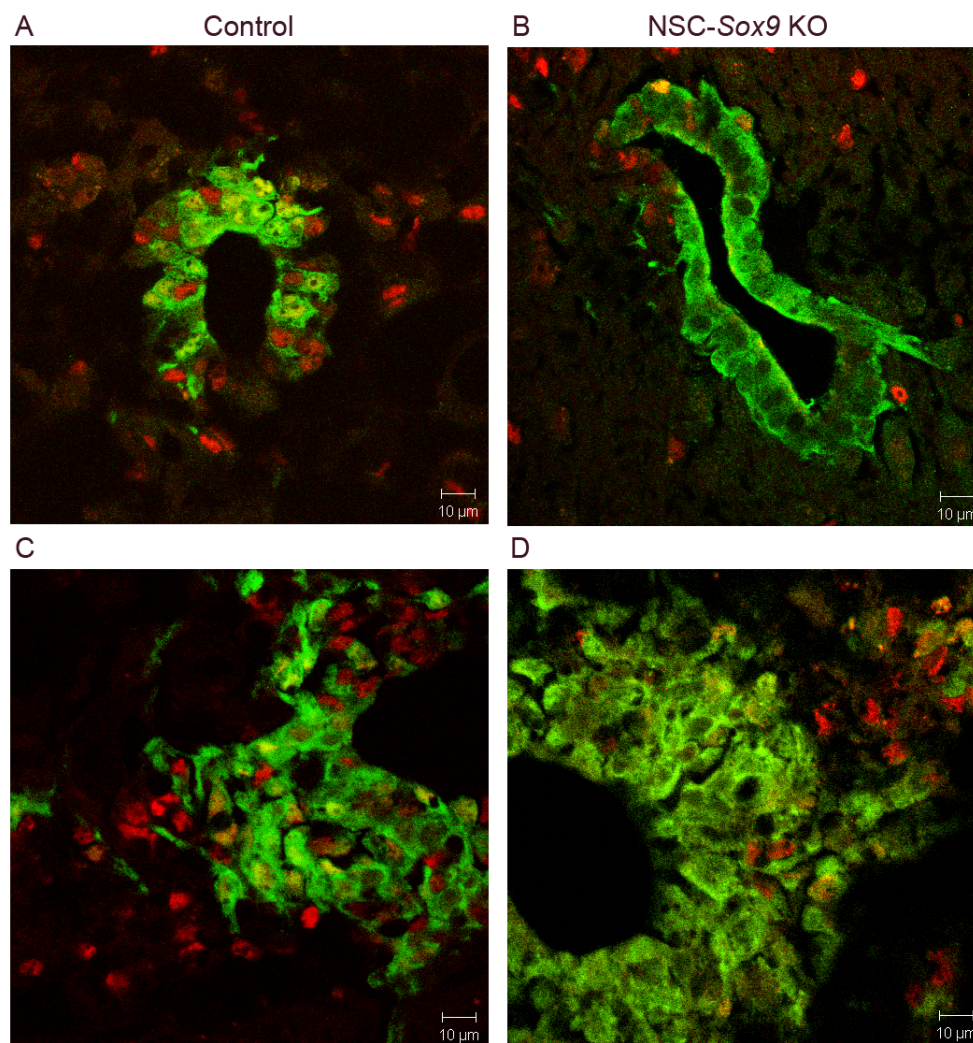
E

Sox9 is Significantly Reduced in NSCs and Their Progeny in NSC Specific Sox9 KO Mice
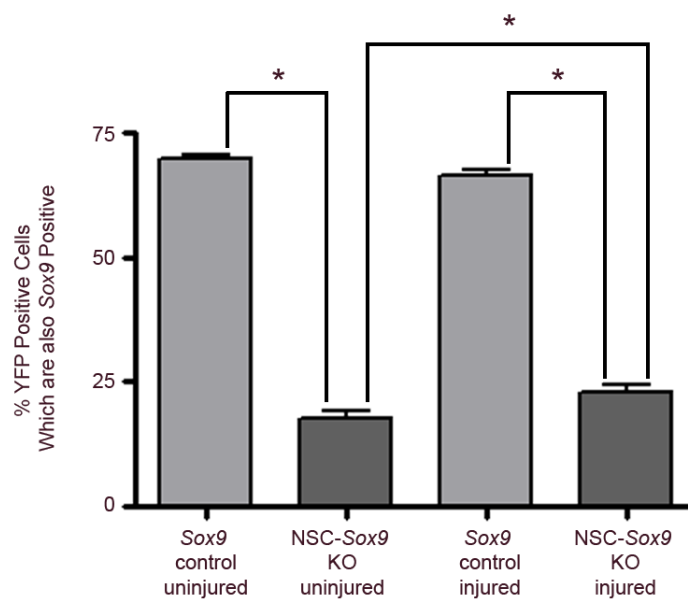
cord injury. To verify that YFP expression in neural stem cells was unchanged by the *Sox9* ablation we first evaluated the frequency of YFP-expressing cells in uninjured areas of the spinal cord (>3.2mm rostral to the epicenter of the lesion). There was no significant difference in YFP labelling of the ependymal layer in spinal cord sections taken from uninjured areas in $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice (Figure 13). There was however significantly more YFP labelled cells in control sections compared to sections from neural stem cell-specific *Sox9* knockout mice when taken from the lesion (4.8mm of the spinal cord centered on the epicenter of the injury). Control mice averaged 2214 YFP+ cells per area of the spinal cord quantified, but the knockout mice only averaged 1127 cells per area quantified. Since only one-tenth of the sections were quantified, for total YFP numbers we estimate that control mice had approximately 22140 neural stem cells and their progeny produced after spinal cord injury, whereas the knockout mice only had 11270. Since there appeared to be many more sections with YFP+ cells outside of the ependymal layer in the control mice we classified the total number of YFP+ cells into three different areas of the injury; at the epicenter of the injury (an area of 0.64mm of spinal cord centered on the epicenter of the injury), rostral to the epicenter (>0.32mm rostral from the epicenter of the injury), and caudal to the epicenter (>0.32mm caudal from the epicenter of the injury). The average number of YFP-expressing cells at the lesion epicenter was not significantly different between the control and the neural stem cell-specific *Sox9* knockout mice (Figure 14). However, there was significantly less YFP+ cells both rostral and caudal to the epicenter of the injury in the knockout mice (Figure 14). This suggests that the reductions in YFP expression in the knockout mice is

Figure 13: *Sox9*-ablated neural stem cells proliferate less following spinal cord injury (SCI). A,B) In uninjured portions of the spinal cord from spinal cord injured mice YFP expression (green) is still contained within the ependymal layer in a wild-type mouse (A) and a neural stem cell-specific *Sox9* knockout mouse (B). C) Quantification of the labelling efficiency of YFP in the ependymal cells of uninjured spinal cord sections showed there was no difference between the control mice and the neural stem cell-specific *Sox9* knockout mice as demonstrated by a student's t-test (p>0.05). D, E) Neural stem cells and their progeny express YFP (green) after they have migrated away from the ependymal layer in a wild-type mouse (D) and a neural stem cell-specific *Sox9* knockout mouse (E) 11 weeks after a spinal cord injury. Scale bars indicate 100μm. F) Quantification of overall YFP expression after spinal cord injury showed there was significantly less YFP+ cells in neural stem cell-specific *Sox9* knockout mice compared to control mice as demonstrated by a student's t-test (p<0.05, N= 9 NSC-*Sox9* KO, 9 controls)

A

B

C
YFP Expression in the
Ependymal Layers of uninjured
Spinal Cord Sections

D

E

F
NSC Proliferation is Reduced
in NSC Specific *Sox9* KO
Mice After SCI
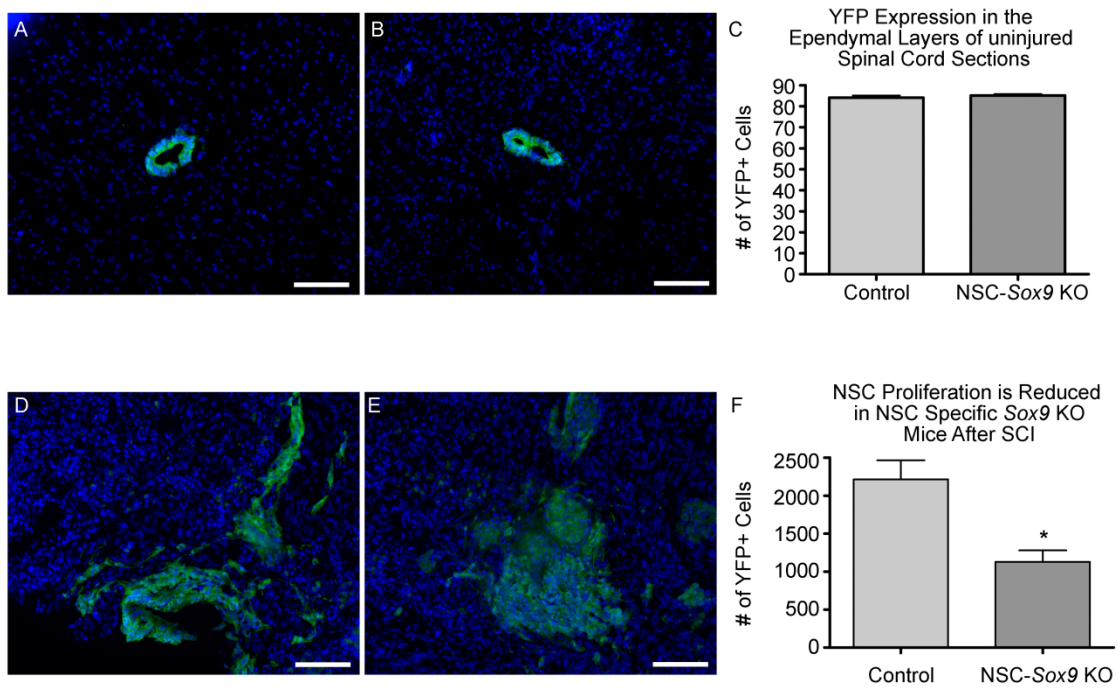
Figure 14: Increased numbers of YFP+ cells in wild-type mice compared to neural stem cell-specific *Sox9* knockout mice is due to a greater number of neural stem cells becoming activated and surviving distant to the lesion epicenter. A-J) Sections of YFP+ (green) neural stem cells and their progeny through an injured spinal cord moving from rostral to caudal over an area of 1.28mm (showing every other section) in a wild-type mouse (A-E) and a neural stem cell-specific *Sox9* knockout mouse (F-J) 11 weeks after spinal cord injury. Scale bars indicate 100µm. K) There was no difference in the number of YFP+ cells at the epicenter of the injury (an area of 0.64mm of spinal cord centered on the epicenter of the injury), but there was a significant reduction in YFP+ cells both rostral and caudal to the epicenter of the injury, as demonstrated by a one-way ANOVA, Bonferroni post-hoc test ($p < 0.05$, N= 9 NSC-*Sox9* KO, 9 controls).
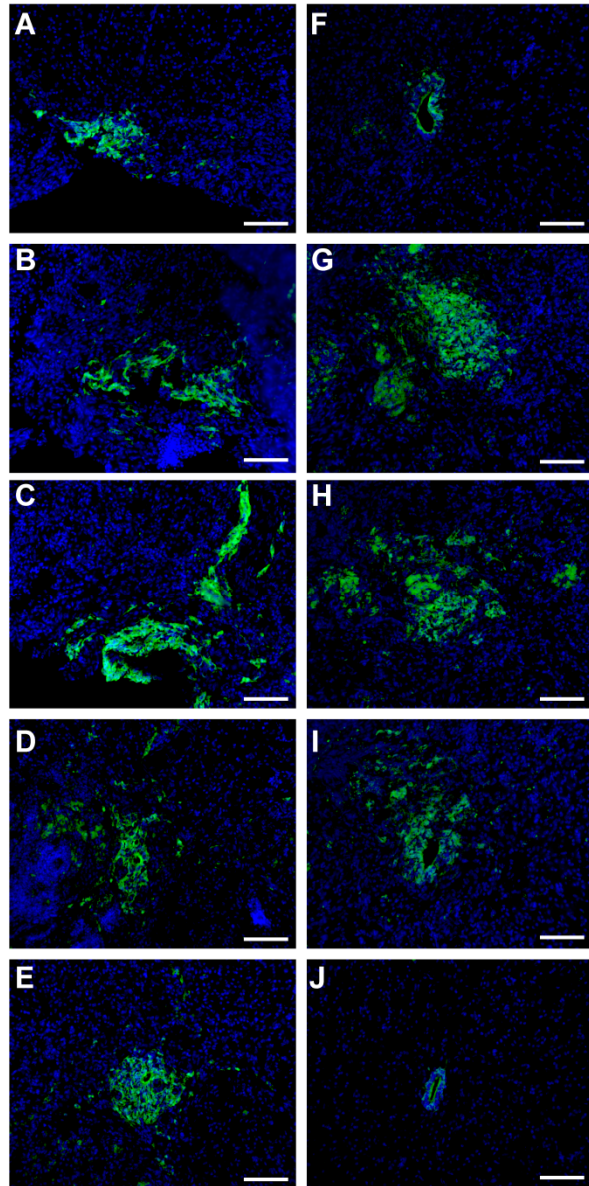
K

There are Less YFP+ Cells Distal to the Lesion Epicenter in NSC-*Sox9* KO Mice
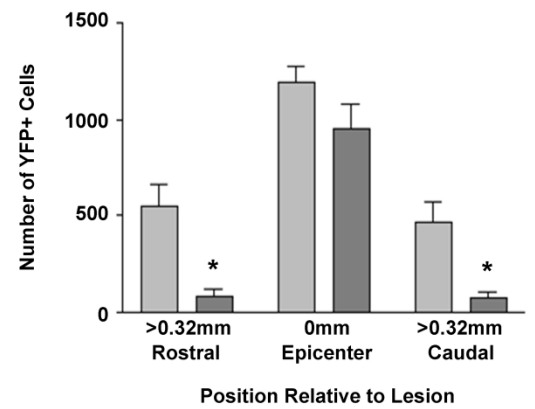
due to decreased cell division in neural stem cells distant from the epicenter of the injury.

3.3.3 Neural stem cells produce fewer astrocytes and oligodendrocytes and more neurons after spinal cord injury in neural stem cell-specific *Sox9* conditional knockout mice

Eleven weeks after injury the mice were perfused and their spinal cords sectioned and double immune-stained for YFP expression and for either GFAP, APC-CC1, DCX or NeuN expression. Double labelling for YFP and the astrocyte marker GFAP revealed a significant decrease in the percentage of YFP+ cells that were also GFAP+ in the neural stem cell-specific *Sox9* knockout mice compared to controls (Figure 15). In control mice an average of 14.6% of their YFP+ cells were also GFAP-expressing whereas in the knockout mice only 12.4% of their YFP+ cells were also GFAP-expressing. This supports the findings in the BrdU experiment that in the absence of SOX9 expression neural stem cells reduce their production of new astrocytes after spinal cord injury. This may not seem like a large difference in new astrocyte production, but since control mice also demonstrated almost twice as much neural stem cell proliferation as knockout mice they produced more than twice as many new astrocytes after spinal cord injury (Figure 15). Double labelling for YFP and the oligodendrocyte marker APC-CC1 also revealed a small but statistically significant reduction in the production of new oligodendrocytes by *Sox9* ablated neural stem cells (~10 newborn oligodendrocytes per knockout mouse versus ~36 in controls) (Figure 16). Double labelling for YFP and the neuroblast marker DCX failed to reveal any neuroblasts in the sections analyzed. Double labelling for YFP and the neuronal marker NeuN revealed a small increase in newborn neurons in the

Figure 15: Neural stem cells generate fewer astrocytes when *Sox9* is knocked out after spinal cord injury. A, B) DAPI staining is blue (i), YFP staining is green (ii), GFAP staining is red (iii) and co-localization of all three markers (iv) indicates newly generated astrocytes after spinal cord injury shown as yellow in a control mouse (A) and a neural stem cell-specific *Sox9* knockout mouse (B). Pictures were taken with a confocal microscope. Scale bars indicate 10µm. C) Quantification of the percentage of YFP+ neural stem cells and their progeny that are also GFAP+ astrocytes. Neural stem cell-specific *Sox9* knockout mice produce a significantly smaller percentage of astrocytes than control mice as determined by a student's t-test ($p<0.05$). D) Quantification of the overall number of new astrocytes (YFP+ and GFAP+) produced throughout the lesion (4.8mm of the spinal cord centered on the lesion epicenter) shows neural stem cell-specific *Sox9* knockout mice produce significantly less astrocytes than control mice as determined by a student's t-test ($p<0.05$, N= 9 NSC-*Sox9* KO, 9 controls).

A Control
B NSC-*Sox9* KO

C

**Knocking out *Sox9* in NSCs
Reduces Astrocyte Production After SCI**

% of YFP Positive Cells
also GFAP Positive

Control   NSC-*Sox9* KO

D

**Significantly Less Astrocytes are
Produced from *Sox9* Ablated NSCs After SCI**

# of new Astrocytes
Produced After SCI

Control   NSC-*Sox9* KO

Figure 16: *Sox9*-ablated neural stem cells generate less oligodendrocytes after spinal cord injury. A) A newly generated oligodendrocyte is YFP+ (green) and APC-CC1+ (red) as indicated by the arrow after spinal cord injury. B) Co-localization of the neural stem cell derived oligodendrocyte from (A) at a higher magnification is shown with DAPI as blue (i), YFP as green (ii), APC-CC1 as red (iii) and co-localization shown in yellow (iv). Pictures were taken with a confocal microscope. Scale bars indicate 10µm. C) Quantification of the total number of new oligodendrocytes found that neural stem cell-specific *Sox9* knockout mice produce significantly less oligodendrocytes than control mice as demonstrated by a student's t-test (p<0.05, N= 9 NSC-*Sox9* KO, 9 controls).
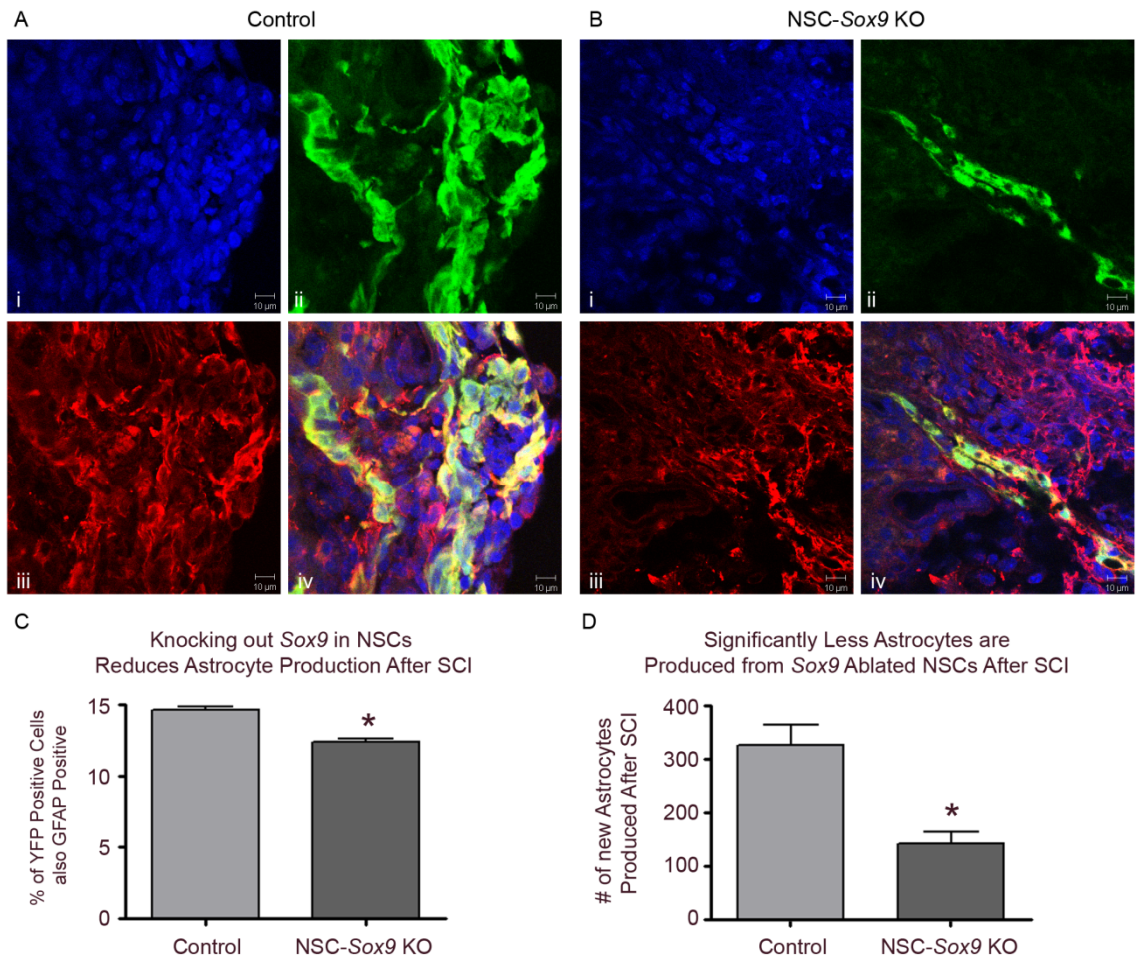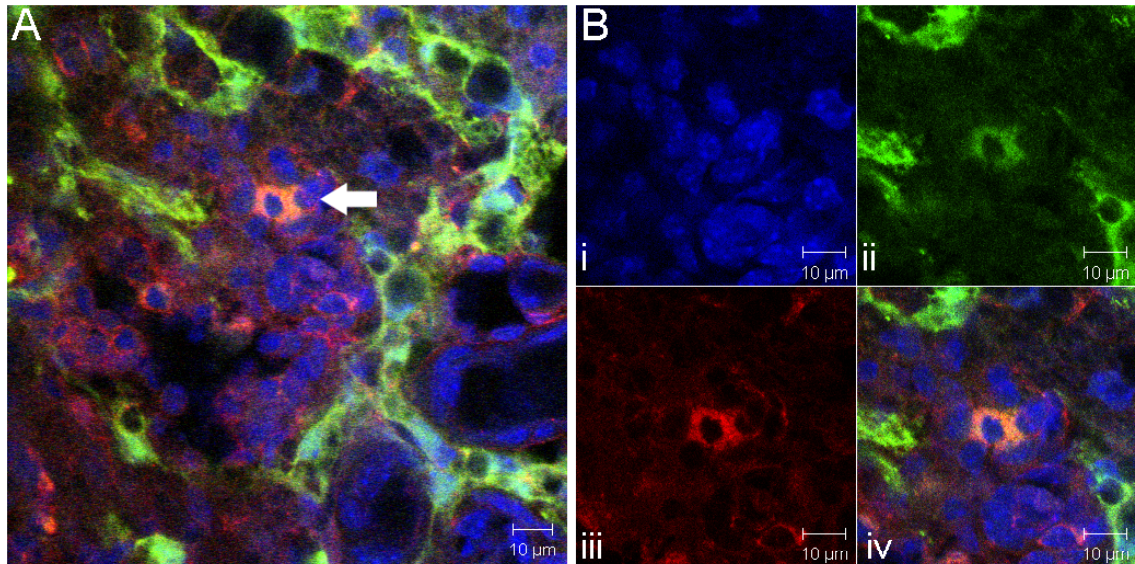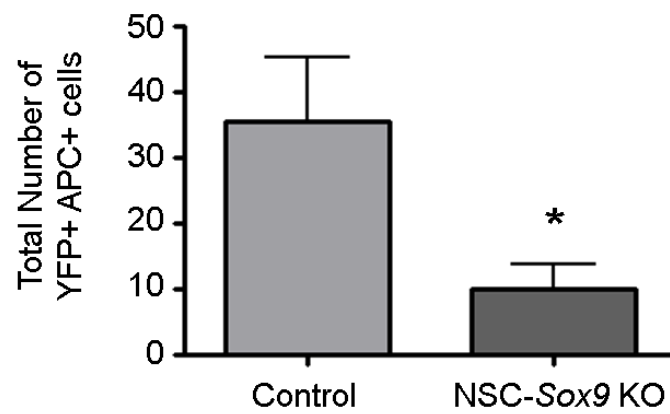
C   Knocking out *Sox9* in NSCs Decreases the
Production of Oligodendrocytes After SCI

sections of neural stem cell-specific *Sox9* knockout mice (~10 per knockout mouse versus zero in controls) (Figure 17).

3.3.4 Locomotor recovery in neural stem cell-specific *Sox9* knockout mice is not improved compared to controls

To determine if knocking out *Sox9* in just the neural stem cells alone is sufficient to improve functional recovery in mice after spinal cord injury we compared the knockout mice to wild-type mice using two behavioural tests to assess their recovery. Tamoxifen was administered to the neural stem cell-specific *Sox9* knockout mice and the wild-type mice for one week followed by one week of no treatment to allow for recombination to take place. The mice received a spinal cord injury at the level of the ninth thoracic vertebrae (T9) with a force of 70kdynes. The next day after injury the Basso Mouse Scale was used to assess the hindlimb function of the mice. Any mouse with an average score above 0.5 the day after surgery was not used in the study. The mice were tested once a week for 11 weeks after injury. There was no significant difference found in the hindlimb recovery between the neural stem cell-specific *Sox9* knockout mice and the control mice at any point during the 11 weeks after injury (Figure 18). The knockout mice had a slightly higher average BMS score at 11 weeks after injury with an average of 1.3 versus 0.65 for the control mice. However, the difference in their scores was not significantly different.

Figure 17: Neurogenesis was increased in neural stem cell-specific *Sox9* knockout mice compared to control mice. A, B, C) YFP staining is shown in green (A), NeuN staining is shown in red (B), and co-localization shows a neural stem cell derived neuron in yellow from a neural stem cell-specific *Sox9* knockout mouse after spinal cord injury (C). This picture was taken with a confocal microscope. Scale bars indicate 10µm. D) Quantification of the total number of new neurons in neural stem cell-specific *Sox9* knockout mice compared to control mice after spinal cord injury. Statistical analysis could not be performed comparing control mice to knockout mice since there were no new neurons found in the control mice (N= 9 NSC-*Sox9* KO, 9 controls).

Figure D: Knocking out *Sox9* in NSCs Results in Increased Neurogenesis Following Spinal Cord Injury
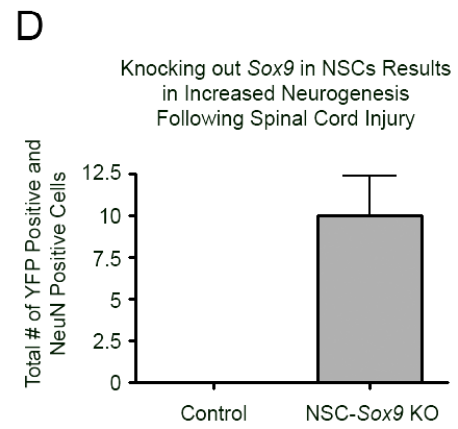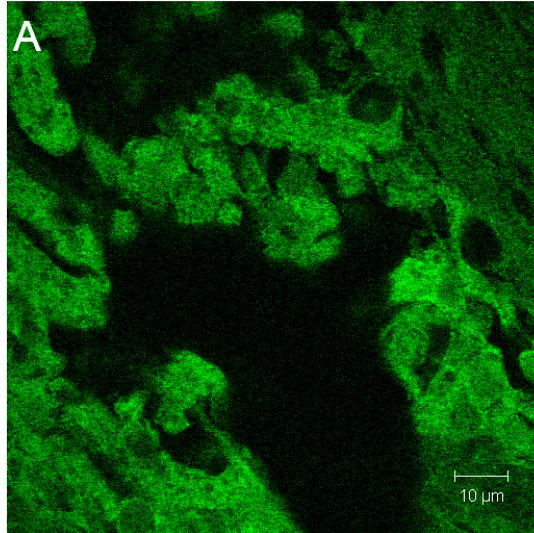
Figure 18: Knocking out *Sox9* in neural stem cells does not improve locomotor function

after spinal cord injury. There is no significant difference in the hindlimb function of

neural stem cell-specific *Sox9* knockout mice and control mice at any week up to 11

weeks after spinal cord injury using the Basso Mouse Scale (BMS), as demonstrated by a

two-way repeated measures ANOVA, (p>0.05), N= 10 NSC-*Sox9* KO, 10 Control

There is no Difference in BMS Scores Between Control and NSC-*Sox9* KO Mice After Spinal Cord Injury
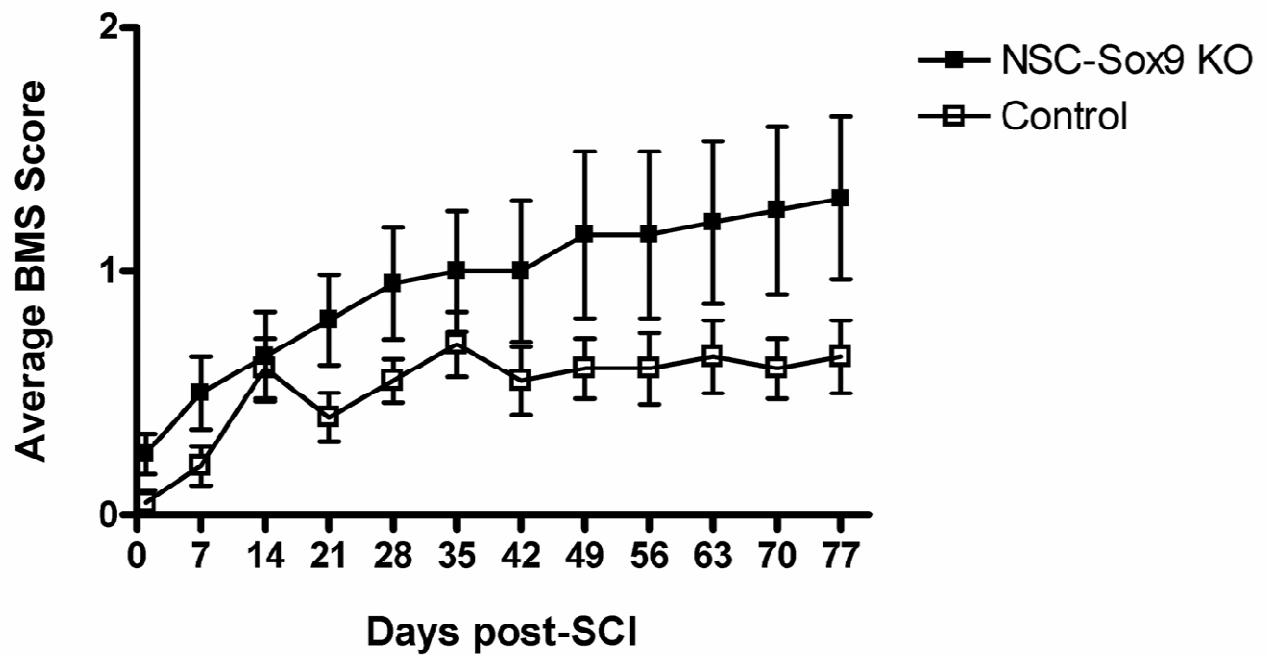
Rodent activity boxes were used to determine the overall distance traversed by a mouse over a two hour period. The mice were tested after *Sox9* had been knocked out with tamoxifen treatment but prior to spinal cord injury, and then again at 4, 8, and 11 weeks after spinal cord injury. There was no significant difference found in overall locomotion between the knockout and the control mice at any of the time points (Figure 19). Both groups of mice had reduced locomotion after injury and it never increased throughout the experiment. This agrees with the results from the BMS testing indicating that neural stem cells without *Sox9* expression are not sufficient to improve functional recovery of the mice on their own.

3.3.5 CSPG levels at the lesion are not affected by neural stem cell-specific *Sox9* ablation

To determine if the reduction in astrocyte production seen in the neural stem cell-specific *Sox9* knockout mice was sufficient to also reduce the level of CSPGs at the lesion, sections from control and *Sox9*-ablated mice were stained with CS-56, an antibody that recognizes many CSPGs (Avnur and Geiger 1984). There was no significant difference in total CSPG levels after spinal cord injury between wild-type mice and neural stem cell-specific *Sox9* knockout mice (Figure 20). Therefore, despite reductions in proliferation and astrocyte production in neural stem cell-specific *Sox9* mice levels of CSPGs were unaltered.

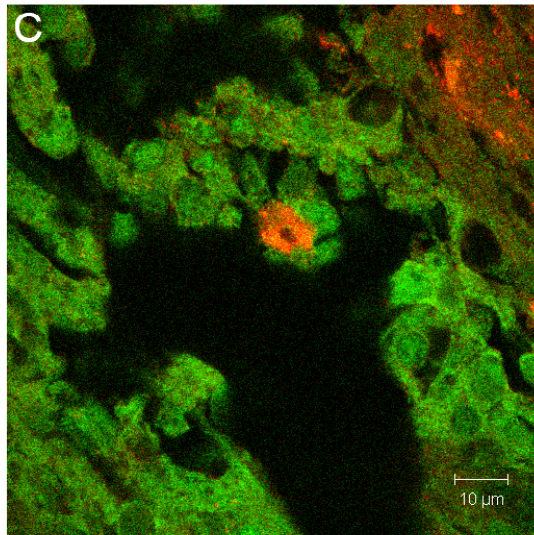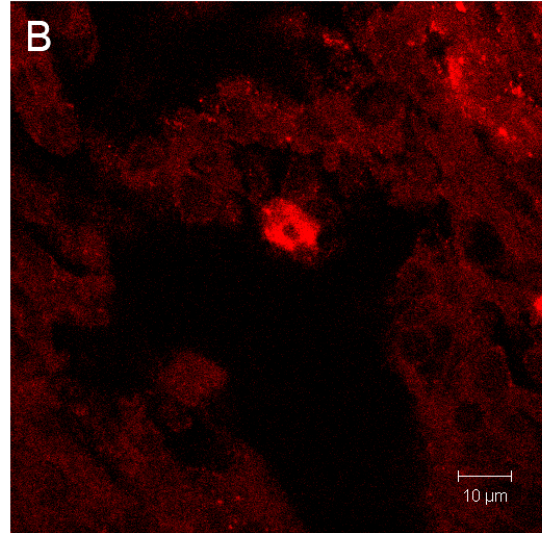Figure 19: Knocking out *Sox9* in neural stem cells does not lead to improved locomotion after spinal cord injury. There is no significant difference between the neural stem cell-specific *Sox9* knockout mice and the control mice in overall distance travelled over a 2 hour period in a rodent activity box (P>0.05, two-way repeated measures ANOVA; N= 10 NSC-*Sox9* KO, 10 controls)

There is no Difference Between Control and NSC-*Sox9* KO Mice in Overall Locomotion After Spinal Cord Injury
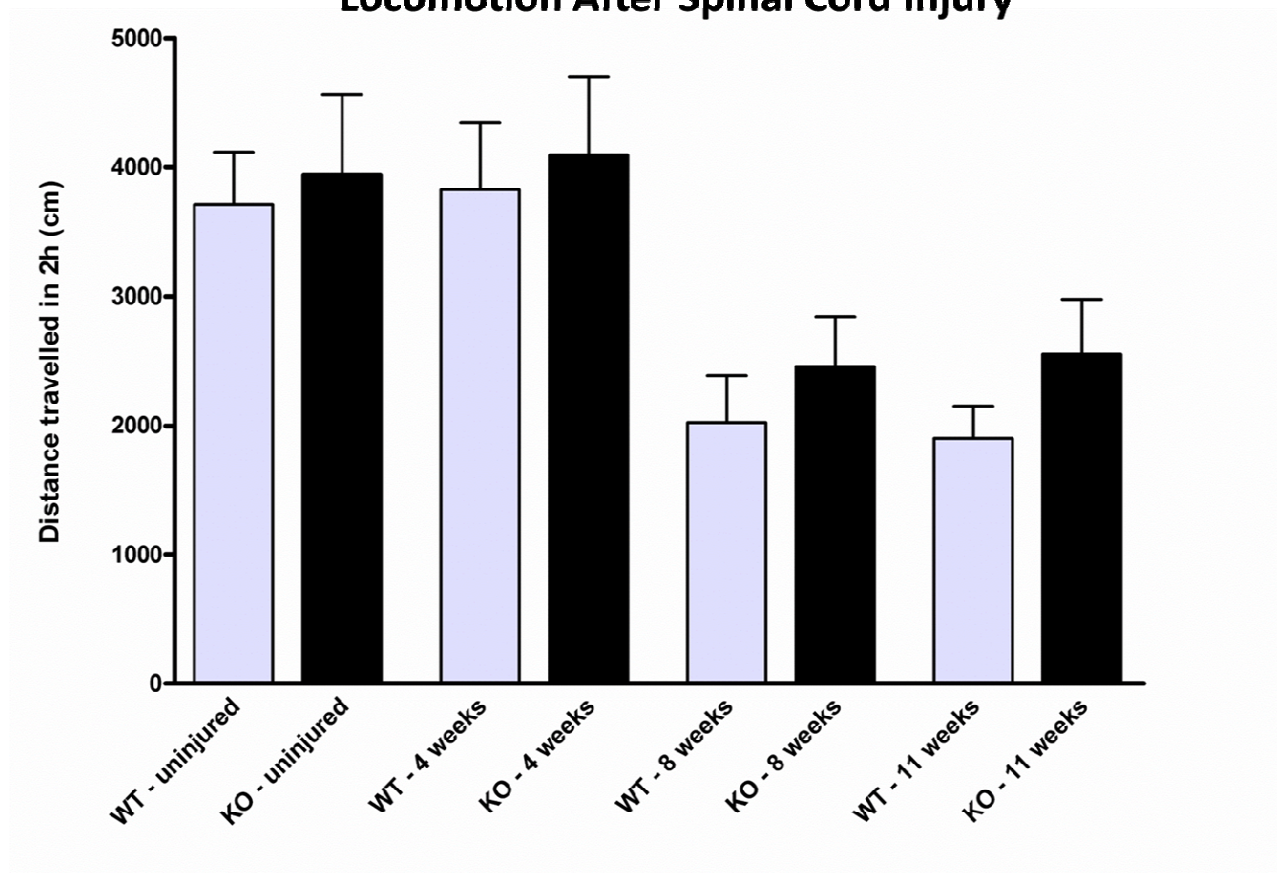
Figure 20: There was no difference in the levels of chondroitin sulfate proteoglycans

(CSPGs) at the lesion between control mice and neural stem cell-specific *Sox9* knockout

mice. A, B) CS-56 staining indicating CSPGs after a spinal cord injury is shown in green

in a wild-type mouse (A) and a neural stem cell-specific *Sox9* knockout mouse (B). Scale

bars indicate 100µm. C) Quantification of CS-56 shows there is no difference in the area

of CS-56 per spinal cord section between control mice and neural stem cell-specific *Sox9*

knockout mice as demonstrated by a student's t-test (p>0.05, N= 9 NSC-*Sox9* KO, 9

controls).

C
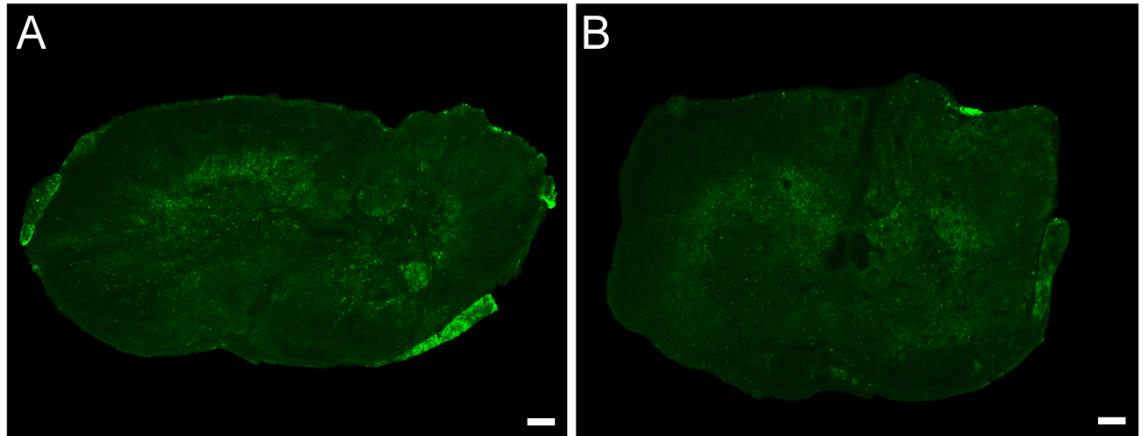
Knocking out Sox9 in NSCs Does Not
Change CSPG levels After SCI
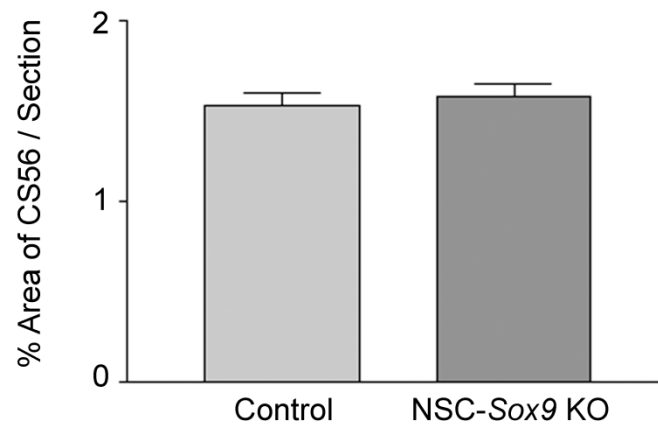
Chapter 4

Discussion:

4.1 Efficiency of the Cre-loxP system as a knockout strategy

The Cre-loxP system was used in the experiment using the *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice to both activate YFP expression by removing a STOP codon in front of the *YFP* gene, and to knockout *Sox9* by removing exons 2 and 3 of the *Sox9* gene. Both recombination events are based on Cre activity, thus they should both occur with the same frequency. Once the Cre-ER fusion protein is allowed to move into the nucleus it should both activate *YFP* and knockout *Sox9*. However, these events do not occur with equal frequencies. In uninjured areas of the spinal cord approximately 90% of the ependymal layer is labelled with YFP (Meletis, Barnabe-Heider et al. 2008). In uninjured portions of the spinal cord in the neural stem cell-specific *Sox9* knockout mice 17.7% of the YFP+ cells in the ependymal layer still express SOX9. If the Cre-ER protein had equal access to the *YFP* and *Sox9* genes we should never see a cell in a knockout mouse that is YFP+ and SOX9+. Even if the two recombination events occurred with equal probabilities, but sometimes only one would happen in a given cell, we should still expect SOX9 to be expressed in less than 10% of YFP+ cells since YFP has a recombination success of 90%, and SOX9 is not expressed in every ependymal cell even in the wild-type mice. This suggests that the Cre-ER protein has easier access to the *YFP* gene than the *Sox9* gene. In the mouse, the ROSA locus, which is where our *YFP* gene was located, is on chromosome 6 (NCBI), and the *Sox9* gene is located on chromosome 11 (NCBI). It is possible that the Cre-ER protein has

easier access to the loxP sites on chromosome 6 than the ones on chromosome 11, and so the *Sox9* knockout efficiency may not be able to be improved due to its position in the chromosome.

4.2 Neural stem cell proliferation

The first objective of my thesis was to determine if knocking out *Sox9* changes neural stem cell proliferation after spinal cord injury. We predicted that after *Sox9* ablation there would be reduced neural stem cell proliferation compared to wild-type mice. We found more YFP+ cells in wild-type, *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice (an average of 2214 cells per 4.8mm of the spinal cord centered on the lesion epicenter) than in the knockout, *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice (an average of 1127 cells per 4.8mm of the spinal cord centered on the lesion epicenter). These results suggest that wild-type neural stem cells produced approximately 2-fold more progeny than *Sox9*-ablated neural stem cells. One caveat to this experiment is that we cannot distinguish if the difference in neural stem cell progeny is due to neural stem cell proliferation or cell death. It is possible that the increased number of YFP+ cells in the *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice is due to decreased cell death. To investigate this possibility we could carry out TUNEL staining to determine if there are more apoptotic nuclei in YFP+ cells in the *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice than in the controls.

By analyzing the distribution of YFP+ cells in *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice we determined that at the epicenter

of the lesion *Sox9*-ablated and wild-type neural stem cells demonstrated approximately the same number of progeny, but YFP+ cells were greater rostral and caudal to the lesion in the control mice. This agrees with previous findings that showed reduced neurosphere formation when neural stem cells are grown in culture without *Sox9* (Scott, Wynn et al. 2010). When neural stem cells are grown in culture they are separated into single cells which then proliferate to form neurospheres. Since they found a 90% reduction in the amount of neurospheres without *Sox9* this indicates a reduction in neural stem cell activity. However, our results indicate that after a spinal cord injury, *Sox9* is only necessary to activate neural stem cells rostral and caudal to the lesion epicenter.

Sox9-ablated neural stem cells may be harder to activate than control neural stem cells due to the absence of extracellular signalling molecules that may propagate an "injury signal" rostral and caudal to the injury. Directly at the injury site neural stem cells are activated in both mouse groups. The impact of the injury, or large amounts of signalling molecules, may be able to override the *Sox9* activation pathway to activate neural stem cells even in its absence. Once activated, these neural stem cells may release extracellular molecules such as cytokines into the surrounding area that activate neural stem cells just outside the lesion. *Sox9* may influence the genes responsible for releasing these molecules or, once nearby neural stem cells bind these molecules *Sox9* may be involved in the pathway these molecules start to then activate the cell. One potential molecule is the cytokine transforming growth factor beta 1 (*TGF-β1*). Previous research has demonstrated that neural progenitor cells from mice express TGF-β1 in cell culture (Klassen, Imfeld et al. 2003), and that levels of TGF-β1 are elevated after a spinal cord injury (Hawryluk, Mothe et al. 2012). *TGF-β1* has also been shown to promote the

production of astrocytes (Stipursky and Gomes 2007), and in chondrocyte studies it has

been shown to increase *Sox9* expression (Xu, Wu et al. 2012). Therefore, one possible

mechanism is that neural stem cells at the lesion epicenter are activated after a spinal cord

injury and secrete TGF-β1 into the surrounding extracellular matrix. TGF-β1 then

activates neural stem cells in the area surrounding the lesion and up-regulates SOX9

expression. SOX9 causes these neural stem cells to proliferate and produce more TGF-

β1, which promotes more astrocyte production and the spreading of neural stem cell

activation. In *Sox9*-ablated neural stem cells TGF-β1 cannot elevate SOX9 expression

resulting in less spread of neural stem cell activation/proliferation. There would be less

TGF-β1 released and so less drive promoting astrocyte production. This model could be

tested by using a TGF-β1 inhibitor, such as suramin sodium (Korrapati, Shaner et al.

2012) after spinal cord injury and measuring neural stem cell activation in

$Sox9^{\text{wild/wild}}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice.

4.3 Neural stem cell differentiation frequency

4.3.1 Glial differentiation

The second objective of my thesis was to determine if *Sox9* ablation alters glial

differentiation from neural stem cells after spinal cord injury. We predicted that after

Sox9 ablation, neural stem cells would reduce their production of glial cells (astrocytes

and oligodendrocytes) after spinal cord injury. Our hypothesis was supported as reduced

astrocyte production was seen two weeks after injury in the ubiquitous *Sox9* knockout

mice using BrdU labelling, and eleven weeks after spinal cord injury in neural stem cell-

specific *Sox9* knockout mice using YFP labelling. In the experiment using BrdU labelling, the percentage of BrdU+ cells that also expressed GFAP was reduced in the ubiquitous *Sox9* knockout mice (9.6% versus 11.7%). Using the *Sox9*$^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice and the *Sox9*$^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* controls, we found that the percentage of YFP+ cells that also expressed GFAP was reduced in the neural stem cell-specific *Sox9* knockout mice (12.4% versus 14.6%). There are two possible reasons that the percentages of astrocytes were higher using YFP labelling than when using BrdU labelling. The first explanation is that the mice were studied at different time points after injury. Some of the labelled neural stem cells or other progenitor cells they produced may have died between two and eleven weeks after injury, whereas the astrocytes survived, making their percentage of the total number of labelled cells higher. The Meletis study did show a small increase in GFAP+ cells between one month and ten months after injury (Meletis, Barnabe-Heider et al. 2008). The other explanation is that BrdU labels all dividing cells, thus some other progenitor cell types may have been labelled, such as oligodendrocyte progenitors which are thought to actively divide in the uninjured cord (Barnabe-Heider, Goritz et al. 2010). The percentage of astrocytes would be less due to these other cell types and their progeny also being labelled with BrdU, but YFP only labels the neural stem cells in the ependymal layer of the spinal cord.

The Frisen lab found a similar percentage of labelled cells becoming GFAP+ astrocytes in wild-type mice as they showed a differentiation frequency around 15% at one month and ten months after spinal cord injury (Meletis, Barnabe-Heider et al. 2008). They used a transection spinal cord injury in their study, indicating the frequency of

astrocyte differentiation from neural stem cells is very similar after transection and contusion spinal cord injuries.

When *Sox9* is knocked out during central nervous system development researchers found that there are almost no astrocytes in the spinal cord at 14.5dpc (Stolt, Lommes et al. 2003). *In vitro* cell culture work has shown when *Sox9* is knocked out there is a one-third reduction in astrocyte production from neural stem cells (Cheng, Pastrana et al. 2009), which is a larger percent reduction than what was found in this thesis. This demonstrates that eliminating *Sox9* expression does not have as strong an effect on reducing astrocyte production in the post spinal cord injury environment compared to cell culture. As discussed in the introduction the spinal cord injury environment strongly promotes astrocyte differentiation even in transplanted neural stem cells (Cao, Zhang et al. 2001; Webber, Bradbury et al. 2007), and it appears this environment strongly promotes astrocyte differentiation from neural stem cells even in the absence of *Sox9*.

Since proliferation of neural stem cells was also greatly reduced in the neural stem cell-specific *Sox9* knockout mouse line there would also be less astrocytes produced due to less neural stem cell division. The overall number of new astrocytes produced after spinal cord injury in the control mice was approximately 3240 (+/- 380 over 4.8mm of the spinal cord centered on the lesion epicenter). The neural stem cell-specific *Sox9* knockout mice produced approximately 1420 (+/- 207 over 4.8mm of the spinal cord centered on the lesion epicenter) new astrocytes. Therefore, the neural stem cell-specific *Sox9* knockout mice produce about half as many new astrocytes after spinal cord injury

compared to controls. Our results suggest that this reduction in newborn astrocytes after injury is not sufficient to alter neurological recovery.

In the experiment conducted with the $Sox9^{flox/flox}$;*FoxJ1*-Cre-ER;*ROSA-YFP* and the $Sox9^{wild/wild}$;*FoxJ1*-Cre-ER;*ROSA-YFP* mice, the knockout mice showed a 72% reduction in oligodendrocyte production compared to control mice. During central nervous system development Stolt et al. reported only 25% as many oligodendrocyte progenitors at 14.5dpc when *Sox9* was knocked out compared to controls (Stolt, Lommes et al. 2003). However, they found that oligodendrocyte progenitor numbers recovered to 73% by 16.5dpc. The authors suggest that the recovery of oligodendrocyte numbers might be due to increased expression of SOX10, that compensates for the loss of SOX9 as SOX10 has been shown to be important in oligodendrocyte development (Stolt, Rehberg et al. 2002). Support for the possibility that SOX10 might be able to compensate for SOX9 loss is provided by the observation that the loss of oligodendrocyte progenitors is reduced to 10% in a *Sox10/Sox9* double knockout mouse (Stolt, Lommes et al. 2003). These researchers argue that *Sox9* is needed for the development of oligodendrocyte progenitors, but once created *Sox10* can compensate for the loss of *Sox9* in the development of mature oligodendrocytes. Therefore, in our *Sox9* knockout mice the decrease in oligodendrocyte numbers is most likely the result of there being a decrease in oligodendrocyte progenitors produced from neural stem cells.

4.3.2 Neuronal Differentiation

The third objective of my thesis was to determine if *Sox9* ablation alters neuronal differentiation from neural stem cells after spinal cord injury. We predicted that *Sox9*-ablated neural stem cells would increase their production of neuroblasts/neurons. In both the experiments with the ubiquitous *Sox9* knockout mice and the neural stem cell-specific *Sox9* knockout mice there were no new neuroblasts produced from the neural stem cells in any of the mouse groups. New neuroblasts and new neurons may have formed, but then subsequently died within two weeks of the injury. While the absence of new neuroblasts or neurons two weeks after injury could be ascribed to the problem of low labelling efficiency of BrdU, the results generated using the *ROSA* reporter strains suggest that this was not the case as YFP labelling is very efficient, and no new neural stem cell derived neuroblasts and very few neurons were observed 11 weeks after spinal cord injury.

There were only an estimated 10 (+/- 2.4) new neurons produced from neural stem cells throughout the lesion in the knockout mice compared to 0 for the wild-type mice. The small number of neurons generated is unlikely to have an effect on neurological recovery, but it does suggest that *Sox9* loss of function may be necessary but not sufficient for neuronal differentiation. We suggest that in addition to loss of *Sox9* a second signal may be required for full neuronal differentiation. Previous research has shown that in *Sox9* deficient mice there is a 30% increase in motor neuron production in the developing central nervous system (Stolt, Lommes et al. 2003), and a two-fold increase in neuron production in cell culture (Cheng, Pastrana et al. 2009). Thus the

putative second signal may be present in the developing spinal cord during neurogenesis, but may be absent in the adult injured (or uninjured) spinal cord.

One signalling pathway that seems to be involved in neurogenesis is the Wnt signalling pathway. Several Wnt proteins (such as Wnt-1, Wnt-3a, Wnt-5a, and Wnt-7a) have been shown to be important for normal brain development (McMahon and Bradley 1990; Lee, Tole et al. 2000), and to promote neurogenesis (Castelo-Branco, Wagner et al. 2003; Hirabayashi, Itoh et al. 2004). The Wnt signalling pathway activates the dishevelled protein (Dvl) which then inactivates glycogen-synthase-kinase-3β (GSK-3β) (van Amerongen and Berns 2006). Activation of GSK-3β has been shown to impair neurogenesis (Eom and Jope 2009), and inhibition of GSK-3β has been shown to increase neurogenesis (Maurer, Bromme et al. 2007). One group of researchers showed that inhibiting GSK-3β was able to improve locomotor functional recovery after a contusion spinal cord injury in rats (Dill, Wang et al. 2008). However, they claimed the improvements were due to improvements in axon sprouting that are inhibited by GSK-3β, and they argue that GSK-3β is activated after spinal cord injury by CSPGs. Since our lab has shown that our ubiquitous *Sox9* knockout mice have reduced CSPG levels (McKillop, Dragan et al. 2013) this could potentially prevent GSK-3β activation which would not only promote axon sprouting but also increase neurogenesis. Perhaps these actions act together to cause functional improvements in our mice and either one alone is not enough to improve recovery. In the neural stem cell-specific *Sox9* knockout mice described herein there was no reduction in CSPG levels, so GSK-3β would have been activated which would inhibit neurogenesis and oppose the effects of the *Sox9* knockout on neural stem cells. Therefore, it is possible that an astrocyte specific *Sox9* knockout mouse would

be sufficient to improve functional recovery, but because it would also influence neurogenesis from the neural stem cells. Other researchers have shown that in chondrocytes, Wnt-3a inhibits SOX9 expression (Thomas, Clarke et al. 2011). Wnt-3a, which is important for normal neurogenesis, is able to inhibit SOX9 which has been shown to inhibit neurogenesis. It is possible that overexpression of Wnt-3a or inhibition of GSK-3β in addition to knocking out *Sox9* may further increase neurogenesis and increase the improvements seen in functional recovery after spinal cord injury.

Previous research has shown that there are sex differences in adult neurogenesis (Pawluski, Brummelte et al. 2009). Differences have been shown between male and female rats in hippocampal neurogenesis in response to stressful conditions. One study argued that chronic stress reduced mature neuronal formation in female rats while not influencing male rats (Hillerer, Neumann et al. 2013). However, other researchers argue that under moderate stressful conditions males show a decrease in neurogenesis while females do not (Pawluski, Brummelte et al. 2009). After a debilitating spinal cord injury, such as the injuries used in this thesis, animals would presumably have increased levels of stress and stress related hormones such as cortisol. The length and levels of this stress may impact neurogenesis from neural stem cells after spinal cord injury depending on the sex of the animal. Although no differences were found between male and female mice in levels of neurogenesis after spinal cord injury in this study, any future research attempting to alter neural stem cells to increase neurogenesis after a spinal cord injury needs to consider the sex of the animals being used, and their stress levels after injury.

4.4 The influence of neural stem cells on functional recovery

The fourth and final objective of my thesis was to determine if knocking out *Sox9* specifically in neural stem cells is sufficient to improve functional recovery after a contusion spinal cord injury. We predicted that *Sox9* ablation would change the behaviour of the neural stem cells to become more beneficial and lead to significant functional improvements. We did not find significant functional improvements in the knockout mice compared to the control mice. The average BMS score was 1.30 for the neural stem cell-specific *Sox9* knockout mice, and it was 0.65 for the control mice eleven weeks after spinal cord injury, but these scores were not significantly different. We have been able to show that knocking out *Sox9* in neural stem cells alone is not sufficient to significantly improve recovery after spinal cord injury, but this does not mean they are not still contributing some benefit to the significant improvements seen in the ubiquitous *Sox9* knockout mice. It is possible that when *Sox9* is knocked out a few different processes are affected that combine to produce the significant functional improvements seen in the ubiquitous *Sox9* knockout mice. For example, it may be that in the absence of SOX9 expression astrocytes at the injury produces less CSPGs, there is a reduction in the invasion of immune cells to the injury site leading to reduced inflammation, and neural stem cells produce less astrocytes. All of these processes reduce growth inhibition at the injury allowing for regrowth and recovery past the injury, but each process on its own is not sufficient to reduce inhibition enough for significant recovery. Thus an astrocyte-specific *Sox9* knockout mouse line might also produce a small benefit to neurological activity, but fail to produce statistically significant results. Evaluating neurological recovery in an astrocyte-specific conditional *Sox9* knockout line of mice would answer

this question. It is important that we determine the cause of the improved recovery after spinal cord injury in our ubiquitous *Sox9* knockout mice to understand how it may be used as a potential therapy to improve the recovery of humans suffering from spinal cord injury.

Future research looking into other potential genes that may alter neural stem cell behaviour using knockout and overexpression strategies is also important as neural stem cells hold the potential to rebuild all of the lost cells and connections from a spinal cord injury. This thesis showed that knocking out *Sox9* in neural stem cells is not sufficient to increase neurogenesis to a large degree. Neurogenesis promoting transcription factors or molecules may also need to be used in order to dramatically increase neuron production. One study argued that when neural stem cells express the transcription factor Olig2 it promotes differentiation of motor neurons, when they only express SOX9 it promotes astrocyte production, and when they express both Olig2 and SOX9 it promotes the production of oligodendrocytes (Esain, Postlethwait et al. 2010). This suggests that if neural stem cells were expressing Olig2, they would either become neurons or oligodendrocytes, depending on their SOX9 expression. These researchers argue that Olig2 expression is regulated by fibroblast growth factor (FGF) signalling. Therefore, using FGFs to increase Olig2 expression in addition to knocking out *Sox9* in neural stem cells may be a more efficient strategy to produce a large amount of neurons, as well as some oligodendrocytes, from neural stem cells after spinal cord injury and improve neurological recovery.

Continuing studies to alter neural stem cell behaviour after spinal cord injury may lead to an understanding of how to replace the lost cells and connections after injury, to help create a therapeutic strategy to improve functional recovery after spinal cord injury.

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007-009-02::6:

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

Curriculum Vitae

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Post-secondary Education and Degrees: The University of Toronto
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B. Sc. Human Biology and Psychology (Honours)
2006-2010

Western Univeristy
London, Ontario, Canada
M. Sc. Anatomy and Cell Biology
2011-2013

Honours and Awards: Western Graduate Research Scholarship
2011-2013

The University of Toronto St. Michael's College silver medal
2010

The University of Toronto St. Michael's College scholarship
2008-2009, 2009-2010

Related Work Experience: Laboratory Teaching Assistant
Human Anatomy, ACB3319
Western University
2011-2013

Laboratory Assistant
Laboratory of Dr. Arthur Brown
Western University
2011

Presentations:

Poster: McDonald, S. Investigation of *Sox9* Ablation on Neural Stem Cell
Behaviour after Spinal Cord Injury. London Health Research Day,
Poster Presentation. 2013.

McDonald, S. Investigation of *Sox9* Ablation on Neural Stem Cell
Behaviour after Spinal Cord Injury. Anatomy and Cell Biology
Research Day, Poster Presentation. Western University. 2012.

Oral: McDonald, S. Investigation of *Sox9* Ablation on Neural Stem Cell
Behaviour after Spinal Cord Injury. Oral Presentation given to
Robarts Data Club, Robarts Research Institute. 2013.

McDonald, S. Investigation of *Sox9* Ablation on Neural Stem Cell
Behaviour after Spinal Cord Injury. Oral Presentation given to a
public audience for the Spinal Cord Injury Open House, Robarts
Research Institute, 2013.