Elucidating the role and mechanisms of CSPGs in modulating endogenous repair processes

in spinal cord injury

By

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Abstract

Populations of oligodendrocytes are susceptible to cell death following spinal cord injury (SCI), which results in axon demyelination. Multipotent resident neural precursor cells (NPCs) and oligodendrocyte precursor cells (OPCs) have the innate potential to replace lost oligodendrocytes, however, their regenerative capabilities are limited within the milieu of SCI. Thus, development of therapies which promote endogenous oligodendrocyte replacement is a critical therapeutic target for SCI repair. Studies by our group have shown that upregulation of chondroitin sulfate proteoglycans (CSPGs) in the extracellular matrix appears to limit oligodendrocytes replacement after SCI. Importantly, it is known that targeting CSPGs can improve functional recovery after SCI. However, the cellular and molecular mechanisms underlying the inhibitory effects of CSPGs remain largely undefined. The discovery of CSPGs specific signaling receptors, leukocyte common antigen-related (LAR) and protein tyrosine phosphatase-sigma (PTPσ), allows us to uncover CSPGs direct mechanisms.

Using *in vitro* models recapitulating the extracellular matrix of SCI, we first identify that CSPGs directly impede the ability of NPCs for proliferation and oligodendrocyte differentiation by signaling through LAR and PTPσ receptors and activation of the Rho/ROCK pathway. Pharmacological inhibition of LAR with Intracellular LAR peptide (ILP) and PTPσ with Intracellular Sigma peptide (ISP) is efficient to block nearly all CSPGs effect on NPCs *in vitro*. Similarly, the presence of CSPGs inhibits OPCs growth, maturation and myelination *in vitro*, which can be overcome by inhibition of LAR and PTPσ receptors.

Capitalizing on these *in vitro* observation, we hypothesized that pharmacological blockage of LAR and PTPσ will promote endogenous oligodendrogenesis following SCI. Using a clinically relevant model of compressive/contusive SCI in the rat, we demonstrate that ILP and ISP play

critical roles in regulating the endogenous cell response to injury. Perturbing LAR and PTPσ signaling attenuates oligodendrocyte apoptosis and myelin damage, while promoting oligodendrogenesis. We unraveled that LAR and PTPσ control oligodendrocyte differentiation partly by modulating microglia response and RhoA activity. In SCI, CSPGs contribute to the pro-inflammatory immune response by signaling through LAR and PTPσ, and that their inhibition forges a pro-regenerative inflammatory landscape characterized by interleukin-10 mediated mechanisms that fosters oligodendrocyte replacement and integrity.

Thus, our findings uncover new roles for CSPGs in regulating secondary injury mechanisms in SCI. We have identified LAR and PTPσ as novel viable targets for modulating immune response and endogenous cell replacement following SCI.

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I have been blessed with many people in my life that have cared and supported

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This thesis is dedicated to my parents

Albert and Patty Dyck

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Contributions of the Authors

Components of five manuscripts published or submitted to peer-reviewed scientific journals (as listed below) are presented in this dissertation. Below is a list of copyrighted material for which permission was obtained:

i) **Scott Dyck**, Arsalan Alizadeh, Santosh Thomas Kallivalappil, Chia-Lun Wu, Evan Proulx and Soheila Karimi-Abdolrezaee. Chondroitin Sulfate Proteoglycans Negatively Modulate the Properties of Adult Spinal Cord Neural Precursor Cells by Signaling through LAR and PTPσ Receptors and Activation of the Rho/ROCK Pathway. *Stem Cells.* 2015 Aug; 33(8): 2550-2563. doi: 10.1002/stem.1979.

My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 90% of the total experimental procedures, data and analysis. Manuscript preparation was done in collaboration with my supervisor. Neural Stem/Precursor Cell cells were provided by Dr. Santhosh Thomas Kallivalappil. PTPσ knockout mice were created by Dr. Chia-Lun Wu and given to us through a collaboration with Dr. Michel Tremblay's Lab. Immunohistochemical analysis shown in figure 3G-J was done by Dr. Arsalan Alizadeh.

ii) **Scott Dyck**, Hardeep Kataria, Bradley Lang, Jerry Silver, and Soheila Karimi-Abdolrezaee. Pharmacological Inhibition of LAR and PTPσ Signaling Promotes Oligodendrocyte Preservation and Replacement following Spinal Cord Injury. *Manuscript will be submitted to GLIA in March 2018.*

I contributed to over 80% of the total experimental procedures, data and analysis, and manuscript preparation. Cell culture of Neural Stem/Precursor Cells was done by Santhosh Thomas Kallivalappil and oligodendrocyte precursor cell cultures were done by Hardeep Kataria.

iii) **Scott Dyck**, Hardeep Kataria[†], Arsalan Alizadeh[†], Kallivalappil T. Santhosh, Bradley Lang, Jerry Silver, Soheila Karimi-Abdolrezaee. Perturbing Chondroitin Sulfate Proteoglycans Signaling through LAR and PTPσ Receptors Promotes a Pro-Regenerative Inflammatory Response following Spinal Cord Injury. *Journal of Neuroinflammation.* Accepted March 2018.

I contributed to over 80% of the total experimental procedures, data and analysis, and manuscript preparation. Intracellular Sigma Peptide (ISP) which was used in this study was created by Dr. Bradley Lang and Dr. Jerry Silver from Case Western University. Cell culture of Neural Stem/Precursor Cells was done by Santhosh Thomas Kallivalappil and microglia cell cultures were done by Hardeep Kataria. Flow cytometry experimental procedures were performed by Hardeep Kataria and myself and data analysis was performed by Arsalan Alizadeh.

_iv)_   **Scott Dyck** and Soheila Karimi-Abdolrezaee. Role of Chondroitin Sulfate Proteoglycans in Development and Pathology of the Central Nervous System. _Experimental Neurology._ 2015 Jul;269:169-187. doi: 10.1016/j.expneurol.2015.04.006.

This review article is a collaborative work between myself and my supervisor highlighting the important role of chondroitin sulfate proteoglycans (CSPGs) in both the developing and pathologic central nervous system. My contribution to this work includes manuscript writing and final approval of manuscript (70% total contribution). I contributed to writing the first draft of the article and my supervisor edited the manuscript. We both prepared the final version of the manuscript.

i)   Arsalan Alizadeh, **Scott Dyck**, Soheila Karimi-Abdolrezaee. Myelin Damage and repair in pathological CNS: challenge and prospects. _Frontiers of Molecular Neuroscience._ 2015 July 27;8:35. doi: 10.3389/fnmol.2015.00035.

This review article highlights a collaboration between myself, Dr. Arsalan Alizadeh and my supervisor. It highlights the pathophysiological events that occurs following damage to myelin in the central nervous system and the various approaches researchers have used to repair and restore myelin damage after insult. I contributed to writing approximately 40% of the manuscript focusing on writing the sections regarding demyelination and its pathological consequences. Dr. Alizadeh wrote the remaining sections covering remyelination strategies.

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List of Abbreviations

ADAMTS4   A disintegrin-like and metalloproteinase with thrombospondint type 1 motif 4

AMPA   α-amino-3-hydroxy-5-methylisoxazole-4- propionate-kainate

ATP   Adenosine triphosphate

cAMP   cyclic adenosine monophosphate

ChABC   Chondroitinase ABC

CNPase   2,3-cyclic nucleotide 3-phosphodiesterase

CNS   Central nervous system

CR3   Complement receptor 3

CREB   cAMP response element binding protein

CRMP2   Collapsing response mediator protein 2

CS   Chondroitin sulfate

CSPGs   Chondroitin sulfate proteoglycans

CSPG-DS   Chondroitin sulfate dissacharides

CST   Corticospinal tract

DC   Dendritic cells

DNA   Deoxyribonucleic acid

DRG   Dorsal root ganglion

EAE   Encephalomyelitis

ECM   Extracellular matrix

EGF   Epidermal growth factor

EGFR   Epidermal growth factor receptor

ELP   Extracellular LAR peptide

FAK   Focal adhesion kinase

FcγR	Fc gamma receptor
FGF	Fibroblast growth factor
FNIII	Fibronectin type III
FOXP3	Forkhead box P3
GAG	Glycoasminoglycan
GAP-43	Growth-associated protein 43
GFAP	Glial fibrillary acidic protein
GalNAc	N-acetyl-D-galactosamine
GlcUA	D-glucuronic acid
hESC	human embryonic stem cells
HSPG	Heparan sulfate proteoglycan
ICC	Immunocytochemsitry
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
ID	Inhibitor of differentiation
IdoA	Iduronic acid
IL	Interleukin
Ig	Immunoglobulin
IGF-1	Insulin like growth factor 1
ILP	Intracellular LAR peptide
IMP	Intracellular Mu peptide
IFN-γ	Interferon-γ
ISP	Intracellular Sigma Peptide

LAR	Leukocyte common antigen-related
LPS	Lipopolysaccharide
M1	Pro-inflammatory macrophage/microglia
M2	Anti-inflammatory macrophage/microglia
MAG	Myelin associated glycoprotein
MAIs	Myelin associated inhibitors
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MMP	Matrix metalloproteinases
MS	Multiple sclerosis
NASCIS	National Acute Spinal Cord Injury Studies
NG2	Neural/glial antigen 2
NgR	Nogo-66 receptor
NMDA	N-methyl-D-aspartate
NPCs	Neural stem/progenitor cells
Nrg-1	Neuregulin 1
OMgp	Oligodendrocyte-myelin glycoprotein
OPCs	Oligodendrocyte precursor cells
PDGF-α	Platelet derived growth factor α
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLP	Proteolipid protein
PNS	Peripheral nervous system

PTPs	Protein tyrosine phosphatases
PTPσ	Protein Tyrosine Phosphate sigma
RNS	Reactive nitrogen species
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
SCI	Spinal Cord Injury
SGZ	Subgranular zone
SVZ	Subventricular zone
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
XT	Xylotransferase
YFP	Yellow fluorescent protein

Chapter 1: Introduction

Chapter 1 is a literature review of data pertinent to this dissertation. It includes some excerpts from two published review articles, which I have previously authored. The contributions for both articles are listed below.

List of copyrighted material for which permission was obtained:

> **Scott Dyck** and Soheila Karimi-Abdolrezaee. Role of Chondroitin Sulfate Proteoglycans in Development and Pathology of the Central Nervous System. *Experimental Neurology.* 2015 Jul;269:169-187. doi: 10.1016/j.expneurol.2015.04.006.
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1.1 Spinal Cord Injury: Overview of Clinical Impact

Over five millennials ago, Smith Papyrus, an Egyptian physician first described spinal cord injury (SCI) as "an ailment not to be treated" as the victims generally died (Eftekharpour et al. 2016). Major advancements in medicine and patient care over the years have changed this dogma, and now the majority of people survive a SCI event (Middleton et al. 2012). SCI occurs following insult to the spinal cord that results in the complete or incomplete loss of motor, sensory and autonomic functions below the level of injury. Depending on the severity and level of injury, the majority of victims are left without the use of their arms and legs, and as a result, they require constant medical care for the rest of their life. These drastic lifestyle changes result in a profound socioeconomic burden for the victim and their family and friends as well as the health care system. Currently, there is no effective therapeutic intervention for SCI. Thus, there is a strong drive to develop new therapeutic interventions to improve the quality of life for those suffering from SCI.

1.1.2 Classification of Human SCI

SCI can be classified as either complete or incomplete (Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016). Complete SCI occurs when there is no spared motor or sensory function below the level of injury, whereas in incomplete SCI there is some preservation of motor or sensory (Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016). The American Spinal Injury Association (ASIA) system is used to classify the extent of injury in humans, where ASIA A represents complete SCI and ASIA B, C or D represents varying degrees of incomplete SCI, with ASIA B being the most severe, and ASIA D being the least severe. The most commonly reported post-SCI neurological score is incomplete tetraplegia (39.5%), followed by complete paraplegia

(22.1%), complete tetraplegia (22.1%) and incomplete paraplegia (16.3%) (Hachem, Ahuja, and Fehlings 2017).

1.1.3 Epidemiology of SCI

According to the National Spinal Cord Injury Statistical Centre, there are 12,500 new cases of SCI each year in North America (Hachem, Ahuja, and Fehlings 2017). The age distribution is bimodal, with a first peak involving young adults and a second peak involving adults over the age of 60 (Stein et al. 2015). Adults older than 60 years of age whom suffer SCI have considerably worse outcomes then younger patients and their injuries usually result from falls, age related bony changes, and side effects from medications (Hachem, Ahuja, and Fehlings 2017). SCI typically affects the cervical level of the spinal cord (50%) with the single most common level affected being C5 (Hachem, Ahuja, and Fehlings 2017). Other injuries include the thoracic level (35%) and lumbar region (11%). With recent advancements in medical procedures and patient care, SCI victims often survive these traumatic injuries and live for decades after the initial injury (Middleton et al. 2012). A recent study published the clinical outcomes of patients who suffered SCI between 1955 and 2006 in Australia and demonstrated survival rates for those suffering from tetraplegia and paraplegia is 91.2% and 95.9%, respectively (Middleton et al. 2012). The 40-year survival rate of these individuals was 47% and 62% for persons with tetraplegia and paraplegia, respectively (Middleton et al. 2012). Today, the estimated life-time cost of a SCI patient is approximately $2.35 million per patient (Hachem, Ahuja, and Fehlings 2017). Therefore, there is a critical need to develop effective repair strategies for this devastating condition.

1.2 Spinal Cord Injury Pathophysiology: An Overview of Primary Injury

SCI typically results from a sudden, traumatic impact on the spine that fractures or dislocates vertebrae. The initial impact of injury is known as primary injury where displaced bone fragments, disc materials, and/or ligaments bruise or tear into the spinal cord tissue (Oyinbo 2011; Dumont et al. 2001). Most injuries do not completely sever the spinal cord (Tator and Fehlings 1991). Secondary injury begins immediately following the initial insult resulting in progressive loss of healthy spinal cord tissue surrounding the lesion site (Oyinbo 2011). Secondary injury starts within minutes after injury and lasts for weeks or months (Oyinbo 2011).

Primary injury results in systemic hypotension, spinal shock, vasospasm, ischemia, plasma membrane compromise, ionic imbalance and neurotransmitter accumulation (Fehlings and Sekhon 2002). Besides prevention, little to nothing can be done to limit the damage that occurs following primary injury, apart from achieving patient's stabilization and decompression of the spinal cord (Fehlings, Vaccaro, et al. 2012). The four characteristic mechanisms of primary injury include 1) Impact plus persistent compression; 2) Impact alone with transient compression; 3) Distraction; 4) Laceration/transection (Dumont et al. 2001). The most common of these types are impact plus persistent compression which typically occurs through burst fractures with bone fragments compressing the spinal cord or through fracture-dislocation injuries (Choo et al. 2007; Dumont et al. 2001; Fehlings, Smith, et al. 2012). Impact alone with transient compression is seen less frequently but most commonly occurs in hyperextension injuries (Dumont et al. 2001). Distraction injuries occur two adjacent vertebrae are pulled apart causing the spinal column to stretch and tear in the axial plane (Choo et al. 2007; Dumont et al. 2001). Lastly, laceration and transection injuries can occur through missile injuries, severe dislocations, or sharp bone fragment dislocations

(Dumont et al. 2001). Laceration and transection injuries can vary greatly from minor injuries to complete transections (Dumont et al. 2001).

1.2.1 Experimental Models of SCI

Many experimental models have been developed to replicate the complex injury processes that occur following SCI (Cheriyan et al. 2014). The aim of these models is to replicate the pathophysiology of SCI in humans as closely as possible. These models vary greatly in terms of the type of animals used, the site of injury, and the injury mechanism. Rats are commonly used in pre-clinical studies as they are inexpensive and they are known to mimic SCI pathophysiology in humans (Metz et al. 2000). The use of mouse models has increased in recent years due to interests in genetic approaches to manipulate the CNS (Jakeman et al. 2000). Larger primate animal models have also been used including marmosets and squirrel monkeys, which better estimate human SCI recovery due to increased ability to assess multiple recovery variables and rehabilitative therapies (Iwanami et al. 2005). Canine (Fukuda et al. 2005) and porcine (Streijger et al. 2016; Lee et al. 2013) models of SCI are also used. In the past, most SCI models have induced injury at the thoracic level of the spinal cord; however, recent studies have begun to focus more on cervical injury models as they have higher clinical relevance given that human SCI most commonly occurs at the cervical level (Cheriyan et al. 2014; Fehlings and Sekhon 2002).

SCI models are categorized by the mechanisms through which they injure the spinal cord; including contusion, compression, distraction, dislocation, transection or chemical models (Cheriyan et al. 2014). Each model and animal type has both pros and cons, which will be discussed in the following sections. Additionally, I will compare the differences we observe in rat versus mouse models of SCI, the two most common animal models used in the literature.

1.2.2 Rat vs Mouse SCI Model

Rat and mouse models of SCI are the most commonly used experimental models. The pathophysiology of rats and mice vary slightly, and it is important to note the differences that exist between the two experimental models when reading the literature. Generally speaking, the rat model of SCI better mimics the pathophysiology of human SCI (Jakeman et al. 2000). In rat (Noble and Wrathall 1985), as well as cat (Blight and Decrescito 1986), monkey (Bresnahan et al. 1976) and human SCI (Fehlings and Sekhon 2002), a cystic cavity forms with a rim of anatomically preserved white matter which surrounds the cavity. Mice are uniquely different where the lesion site becomes filled with dense fibrous connective tissue (Jakeman et al. 2000; Ma et al. 2001; Kuhn and Wrathall 1998; Byrnes, Fricke, and Faden 2010). The presence of fibroblast like cells expressing fibronectin, collagen, CD11b, CD34, CD13 and CD45 is observed within the lesion core of mice, which is absent in rats (Sroga et al. 2003). Another key difference between rat and mice SCI is the time point of inflammatory cell infiltration. Microglia/macrophage infiltration is relatively consistent between rat and mouse models of SCI (Sroga et al. 2003), however, there is a temporal difference in infiltration of neurtrophils and T cells between the two species (Taoka et al. 1997; Sroga et al. 2003). Infiltration of neutrophils, the first responders following injury, peaks at 6 hours post injury, and these levels decline 24-48 hours following SCI in rats (Taoka et al. 1997). Similarly, in mouse SCI, neutrophil infiltration occurs within 6 hours following injury, however their numbers continue to rise and do not peak until 3-14 days post injury (Kigerl, McGaughy, and Popovich 2006). T cell infiltration also varies between rat and mouse SCI models. In rats, T cell infiltration occurs between 3 and 7 days post injury and declines by 50% over the next 2 weeks (Sroga et al. 2003), where as in mice, T cell infiltration is not evident until 14 days post injury and these numbers double between 2 and 6 weeks post injury (Sroga et al. 2003).

1.2.3 Transection Models of SCI

Transection models have been utilized extensively in the literature. They are particularly beneficial for assessing axonal regeneration and subsequent functional recovery following injury (Cheriyan et al. 2014). However, these models do not reflect the complex pathophysiology that occurs following SCI because spinal cord transection are much less common in human SCI. Transection models can either be complete or a partial transection (Cheriyan et al. 2014). The complete transection model is relatively easy to perform and can be used in multiple animal models include rats, mice, dogs and primates (Kwon, Oxland, and Tetzlaff 2002). It involves a laminectomy followed by complete severing of the spinal cord (Cheriyan et al. 2014). Partial transections are also commonly used in the literature. Hemisection lesions (partial transections) involve selectively lesioning the spinal cord and can be used to examine the impact of different spinal tracts on locomotor function and recovery from SCI (Cheriyan et al. 2014). Examples of hemisection lesions that are commonly used in the literature include dorsal hemisection and lateral hemisection models (Arvanian et al. 2009; Dusart and Schwab 1994; Hains et al. 2003). Partial transections are more likely to be seen in human SCI (Cheriyan et al. 2014). This model of SCI is also advantageous as one can compare the ipsilateral lesion to the uninjured contralateral side of the spinal cord in the same animal (Cheriyan et al. 2014). However, partial transections are significantly more difficult to perform than full transections as it can be difficult to determine whether the targets tract is completely severed and as a result, experimental findings can be difficult to reproduce (Cheriyan et al. 2014).

1.2.4 Contusion Models of SCI

The contusion model is the most commonly used injury model in the SCI research field (Allen 1911; Cheriyan et al. 2014; Gruner 1992; Jakeman et al. 2000; Stokes and Reier 1992). Contusion SCI models inflict a transient, acute injury to the spinal cord (Cheriyan et al. 2014). The majority of contusion models involve a weight drop apparatus, which drops onto the spinal cord. The weight drop method requires a laminectomy at the level of injury followed by stabilization of the spinal cord by clamping the vertebrae on both sides of laminectomy (Cheriyan et al. 2014). Allen was the first to develop the weight-drop contusion model in 1911 (Allen 1911). Multiple devices have been created since then which can damage the spinal cord with consistent reproducibility resulting in graded severity depending on the force applied to the cord (Gruner 1992; Jakeman et al. 2000; Stokes and Reier 1992; Cheriyan et al. 2014). One major flaw of the contusion model systems is that it lacks consistent compression of the spinal cord for an extended duration of time and therefore does not replicate the most common form of SCI in humans (Cheriyan et al. 2014; Dumont et al. 2001).

1.2.5 Compression Models of SCI

Compression models of SCI are also used extensively in rodent models. These models provide prolonged compression to the spinal cord. Multiple compression models have been developed for SCI including clip compression (Joshi and Fehlings 2002; Rivlin and Tator 1978b), calibrated forceps compression (Blight 1991; Plemel et al. 2008), and balloon compression (Vanicky et al. 2001; Tarlov, Klinger, and Vitale 1953).

Clip compression is the most commonly used compression model (Joshi and Fehlings 2002; Rivlin and Tator 1978a; Poon et al. 2007; Cheriyan et al. 2014). This is the model, which is

used in Dr. Karimi's laboratory and was also used for all *in vivo* assessment in this thesis. Following laminectomy, a modified aneurysm clip is closed around the circumference of the spinal cord for a set duration of time (usually 1 minute) (Rivlin and Tator 1978a; Poon et al. 2007). These clips are calibrated to deliver a specific force to the spinal cord and have been shown to produce a graded injury depending on the force applied (Rivlin and Tator 1978b). A 50g clip typically produces a severe SCI and 35g clip a moderate-severe injury (Poon et al. 2007). Aneurysm clips were first developed for use in rats but have recently been modified for use in mice (Joshi and Fehlings 2002) as well as in pig models of SCI (Lee et al. 2013). An advantage of the clip compression model is that it provides both compression and contusion to the spinal cord (Cheriyan et al. 2014). The clip compression model has both an acute phase of injury (i.e. contusion) where we see a direct impact to the spinal cord when the clamp is initially closed following by a 1 minute 'compression' stage before the clip is released from the spinal cord (Cheriyan et al. 2014). A major flaw in this model is that there is no way to determine the velocity of the clip upon contact with the cord and thus the actual force delivered to the cord during compression is not accounted for (Cheriyan et al. 2014).

Other compression models have been used but less frequently. Calibrated forceps compression has been shown to produce a graded injury (Blight 1991). Following a laminectomy, a forceps with a specific width, using a space, is compressed around the spinal cord for a selected duration (Blight 1991). This method is relatively inexpensive and has been shown to produce a consistent injury, however, it lacks the acute impact component that we see following SCI and therefore does not mimic the same pathophysiological events we see in acute human SCI (Cheriyan et al. 2014). Similarly, balloon compression models have been used in dogs (Tarlov, Klinger, and Vitale 1953) and more recently in rats (Vanicky et al. 2001). A

catheter is inserted with a small inflatable balloon into the epidural space. The balloon is then inflated to a fixed volume which compresses the spinal cord. Much like the calibrated forceps model, this model is easily reproducible and inexpensive but lacks acute injury to the spinal cord (Cheriyan et al. 2014).

In conclusion, each experimental SCI model has advantages and disadvantages, however the rat contusion and compression models represent the most clinically relevant models available.

1.3 Spinal Cord Injury Pathophysiology: Secondary Injury Mechanisms

Immediately following injury, we observe a host of environmental changes within the spinal cord tissue that ultimately lead to tissue destruction and cell death (Tator and Fehlings 1991). This process is known as secondary injury mechanisms. Secondary injury can be divided into an acute, sub-acute, and chronic phase. The acute phase begins immediately following SCI and includes ionic imbalance, neurotransmitter accumulation (excitotoxicity), free radical formation, calcium influx, lipid peroxidation, inflammation, oedema, and necrotic cell death (Tator and Fehlings 1991; Dumont et al. 2001; Fehlings and Sekhon 2002; Choo et al. 2007; Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016). As the injury progresses, the sub-acute phase of injury begins which involves oligodendrocyte apoptosis, demyelination of surviving axons, Wallerian degeneration, axonal dieback, and the formation of a glial scar around the injury site (Fehlings and Sekhon 2002; Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016; Tator and Fehlings 1991; Dumont et al. 2001; Choo et al. 2007; Oyinbo 2011). As SCI evolves, further changes occur in the chronic phase of injury notably the formation of a cystic cavity, progressive axonal die-back and maturation of the glial scar (Wilson and Fehlings 2011; Rowland et al. 2008). Extensive work has been performed to understand the complex processes that occur following injury, and identify strategies

to limit or modulate the secondary injury cascade in a hope to promote repair after SCI. Here, I will discuss the key components of secondary injury that contribute to the pathophysiology of SCI.

1.3.1 Vascular Insult and Ischemia

SCI is associated with vascular insult which begins at the time point of initial impact. Small diameter blood vessels, mainly capillaries, are most commonly damaged after injury at both the injury site and areas rostral and caudal (Dumont et al. 2001). This results in small areas of hemorrhage within the lesion site which eventually progresses to hemorrhagic necrosis over time (Dumont et al. 2001). Ischemia also occurs after injury. In rat and monkey models of SCI, there is a progressive reduction in blood flow at the lesion epicenter within the first few hours after injury which remains low for up to 24 hours (Rivlin and Tator 1978b; Sandler and Tator 1976). The mechanism behind this is unknown but is thought to occur due to a multitude of factors including hemorrhages, vasospasms, endothelial swelling and disruptions in auto-regulatory homeostasis (Dumont et al. 2001; Sekhon and Fehlings 2001).

The gray matter is more severely affected by acute SCI than peripheral white matter due to the differences in vascularity and reperfusion after injury (Sekhon and Fehlings 2001). The gray matter normally received 3 times the blood flow as the white matter due to its higher metabolic demand and thereby is more vulnerable to vascular disruption (Hayashi et al. 1983). White matter blood flow typically returns to normal levels within 15 minutes post injury, whereas in the gray matter there are multiple hemorrhages and as a result re-perfusion usually does not occur for at least the first 24 hours (Balentine 1978; Sekhon and Fehlings 2001; Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016). Vascular insult, including hemorrhage and ischemia, ultimately leads to cell death and tissue destruction through multiple mechanisms, including oxygen deprivation,

loss of adenosine triphosphate (ATP), excitotoxicity, ionic imbalance, free radical formation, and necrotic cell death (Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016; Fehlings and Vawda 2011).

1.3.2 Oxidative Stress

Following SCI, a significant increase in the production of free radicals occurs which results in oxidative stress and contributes to secondary injury (Sharma et al. 2005). Free radicals are molecules which are highly reactive due to their extra electron in their outer orbit (Dumont et al. 2001). Normally, when free radicals are produced, the endogenous sources of anti-oxidants (i.e. glutathione, superoxide dismutase, thioredoxin) restores these reactive free radicals back to a non-reactive state (Valko et al. 2007). However, in cases such as injury, where free radical levels are elevated, the anti-oxidant pool depletes resulting in oxidative stress (Dumont et al. 2001). Superoxide (O_2^-) is a common free radical formed in the mitochondria. Normally, it is converted to H_2O_2 by superoxide dismutase which then is converted to H_2O and O_2 by catalase (Valko et al. 2007). However, in the presence of iron, H_2O_2 can be converted to highly reactive OH^-. Iron level is much higher after SCI due to its release from hemoglobin in an acidic environment (Dumont et al. 2001).

Oxidative stress can result in damage to the cellular proteins, deoxyribonucleic acid (DNA), and the phospholipid bilayer (Dumont et al. 2001). When free radicals donate their extra electron to polyunsaturated fatty acid it can start a process known as lipid peroxidation (Topsakal, Erol, et al. 2002). Lipid peroxidation destabilizes the phospholipid bilayer causing disruption in ionic homeostasis, and even cell lysis (Hall 2001). Lipid peroxidation usually begins within 5 minutes after injury and is marked by oxidation products such as malonyldialdehyde and 4-

hydroxynonenal (Dumont et al. 2001). These by-products, in conjunction with reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to be neurotoxic, limit mitochondrial function, and inhibit the Na^+/K^+ ATPase pump's function (Dumont et al. 2001).

1.3.3 Imbalanced Homeostasis and Glutamate Excitotoxicity

Ionic homeostasis is altered after SCI and contributes to cellular loss and secondary injury. Lack of ATP, disruption in the Na^+/K^+ pump, increased permeability of the phospholipid bilayer, and glutamate excitotoxicity all contribute to the alterations in intracellular Na^+, K^+, and Ca^{2+} (Dumont et al. 2001; Mataliotakis, Bounakis, and Garrido-Stratenwerth 2016). Glutamate excitotoxicity can be defined as excessive activation of glutamate receptors leading to neuronal injury (Dumont et al. 2001; Park, Velumian, and Fehlings 2004). Following SCI, glutamate, a major excitatory neurotransmitter, is released excessively in and around the injury site and can cause direct damage to spinal cord tissue as well as indirect damage through the production of ROS and RNS (Dumont et al. 2001; Park, Velumian, and Fehlings 2004). Glutamate activates both *N*-methyl-D-aspartate (NMDA) and AMPA-kainate (α-amino-3-hydroxy-5-methylisoxazole-4- propionate-kainate) receptors resulting in excessive influx of Na^+ and Ca^{2+} into the cells (Park, Velumian, and Fehlings 2004). To counteract the initial influx of intracellular Na^+, water enters the cell causing swelling and the cell begins to transport Na^+ back out of the cell in an energy dependant process. Unfortunately, the ability to export intracellular Na^+ is compromised after injury due to limited ATP supply and dysfunction of the Na^+/K^+ ATPase pump (Park, Velumian, and Fehlings 2004; Dumont et al. 2001; Sahuquillo, Poca, and Amoros 2001; Obrenovitch 1999). As a result, accumulation of intracellular Na^+ eventually leads to cytotoxic edema. In addition, the influx in intracellular Ca^{2+} into neurons also contributes to excitotoxic cell death (Park, Velumian, and

Fehlings 2004; Dumont et al. 2001; Abramov and Duchen 2010) by interfering in mitochondrial function and activation of calcium dependant proteases and lipases (i.e. calpains and cyclooxygenase) which can degrade structural proteins and break down the cell membrane (Abramov and Duchen 2010). Influx of Ca^{2+} in the CNS is thought to be the "final common pathway of toxic cell death" (Dumont et al. 2001). Neurons and oligodendrocytes are particularly vulnerable to glutamate excitotoxicity as they express both AMPA and NMDA glutamate receptors (Park, Velumian, and Fehlings 2004; Park, Fehlings, and Liu 2003).

1.3.4 Cell Death: Necrosis and Apoptosis

Both neurons and oligodendrocytes are vulnerable to cell death after injury (Beattie et al. 2002). Cell death typically occurs through two major mechanisms, necrosis and apoptosis. Necrotic cell death occurs immediately following injury affecting primarily neurons and oligodendrocytes (Beattie et al. 2002). Necrosis occurs due to a multitude of factors including accumulation of toxic blood components (Juliet et al. 2009), glutamate excitotoxicity (Gudz, Komuro, and Macklin 2006), ATP depletion (Matute et al. 2007), pro-inflammatory cytokine release by neutrophils and lymphocytes (Antel et al. 1994; Takahashi et al. 2003), and free radical formation (Beattie et al. 2002; Beattie, Farooqui, and Bresnahan 2000; Jana and Pahan 2007; Thorburne and Juurlink 1996). Necrosis is an energy independent process in which the cell swells and eventually lyses spilling its cellular contents into the extracellular matrix (ECM) which results in the initiation of an inflammatory response (Beattie et al. 2002). On the contrary, apoptosis is an energy dependant process that involves programmed cell death through activation of caspases (Beattie, Farooqui, and Bresnahan 2000). In apoptosis, the cell shrinks and is eventually phagocytosed without induction of an inflammatory response (Beattie, Farooqui, and Bresnahan 2000). Apoptosis

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typically occurs in a delayed manner in areas more distant to the injury site and most abundantly affects oligodendrocytes. Loss of over 90% of oligodendrocytes at the lesion epicenter occurs by 7 days after injury (McTigue, Wei, and Stokes 2001). Interestingly, apoptotic cell death even occurs in the chronically injured spinal cord in rat, monkey and human models of SCI, which is thought to be due to loss of trophic support from degenerating axons (Crowe et al. 1997; Guest, Hiester, and Bunge 2005).

In addition to cell death through necrosis and apoptosis, cellular loss can occur through dysregulation of autophagy after SCI. Autophagy is a protective mechanism through which cells degrade cytoplasmic proteins and organelles in a lysosome dependent manner (Mizushima and Komatsu 2011; Mizushima et al. 2008). Recent evidence shows that autophagy is impaired as early as one day following SCI (Liu et al. 2016). Normally, autophagy plays an important role in maintaining the homeostasis of cells by aiding in the normal turnover of proteins and organelles (Mizushima and Komatsu 2011). Autophagy occurs through the formation of an autophagosome which contains potentially toxic proteins and damaged organelles which then fuses with a lysosome to allow for degradation of their contents (Mizushima and Komatsu 2011). The process of fusion between the autophagosome and lysosome and the degradation of the autophagosome's contents is known as autophagy flux (Mizushima and Komatsu 2011). In response to cell injury and endoplasmic reticulum (ER) stress, autophagy is activated and limits cellular loss (Xu et al. 2013; Ogata et al. 2006). Blocking autophagy flux has been associated with neurodegenerative disease including Parkinson's and Alzheimer's disease (Mizushima et al. 2008; Rubinsztein 2008). After SCI, autophagy flux is also disrupted and is thought to contribute to neuronal cell loss (Chen et al. 2015). One day post-SCI a significant increase in the accumulation of autophagosomes occurs within ventral horn motor neurons and dorsal column oligodendrocytes (Liu et al. 2016).

In particular, motor neurons with impaired autophagy flux also express ER-stress-associated initiator caspase 12 indicating that disruption in autophagy is associated with neuronal cell loss after injury (Liu et al. 2016).

1.3.5 Neuroinflammation

Inflammation is a complex process which was originally thought to be detrimental following SCI (Popovich et al. 2001). However, today it is well accepted that inflammation can be both beneficial and detrimental following SCI, depending on the time point and activation state of immune cells (Miron and Franklin 2014). There are multiple cell types involved in the inflammatory response following injury including neutrophils, resident microglia, dendritic cells (DCs), blood-born macrophages, B-lymphocytes, and T-lymphocytes (Donnelly and Popovich 2008). The first phase of inflammation (0-2 days post injury) involves the recruitment of resident microglia in the CNS and blood-born neutrophils to the injury site (Jones, McDaniel, and Popovich 2005). The second phase of inflammation begins approximately 3 days post injury and involves the recruitment of blood-born macrophages, B-lymphocytes, and T-lymphocytes to the injury site (Kigerl et al. 2009; Donnelly and Popovich 2008; Jones, Hart, and Popovich 2005; Beck et al. 2010). The final stage of inflammation involves resolution of inflammation and the formation of a glial scar (Fitch and Silver 2008). It is important to note that chronic inflammation continues well beyond the initial injury impact and may persist for the remainder of an individual's life (Beck et al. 2010). Here, I will discuss the role each cell type plays in the pathophysiology of SCI.

1.3.5.1 Neutrophils

Following SCI, neutrophils are recruited to the spinal cord from the bloodstream by chemoattractant such as interleukin (IL)-8 and interferon-γ (IFN-γ) (Neirinckx et al. 2014). In rat SCI, they migrate across the endothelial barrier and their numbers peak around 6 hours post injury (Taoka et al. 1997). Their levels decline 24-48 hours post injury and by 3 days neutrophils are absent from the injury site (Taoka et al. 1997). They are thought to exacerbate the injury process by damaging neurons, glial cells and endothelial cells by release of toxic ROS and proteases (Dumont et al. 2001). Generally speaking, drugs which reduce neutrophil infiltration after SCI typically result in lower expression of pro-inflammatory cytokines and improved functional outcomes (Neirinckx et al. 2014). It is important to note that neutrophils do have some beneficial effect after injury as ablating the neutrophil population in mice is associated with reduced functional recovery (Stirling et al. 2009; Ghasemlou et al. 2010; Neirinckx et al. 2014).

1.3.5.2 Resident Microglia

In the normal spinal cord, microglia reside amongst other glial cells and play an important role in maintaining tissue homeostasis, phagocytosis of dead cells, and surveying the CNS for any signs of trauma or infection (Hsu et al. 2006). Following SCI, they are activated by a host of factors including outward K^+ currents, ATP, astrocytic connexin hemichannels and various cytokines (David and Kroner 2011). They migrate towards the lesion site at 1.25μm/minute and form a dense border around the lesion preventing further progression of the lesion towards healthy neighbouring tissue (Davalos et al. 2005). They initially release a host of pro-inflammatory cytokines including TNFα and IL-1β which promote the recruitment of blood-born macrophages and lymphocytes to the injury site (David and Kroner 2011). After injury, it is difficult to distinguish resident microglia

from blood-born macrophages without the use of genetic labelling, and therefore, I will discuss the role microglia/macrophages play in SCI collectively (David and Kroner 2011).

1.3.5.3 Blood-born Macrophages

Macrophage are recruited to the injury site around 3 days post injury and their numbers peak at approximately 7 days (David and Kroner 2011; Hsu et al. 2006). They serve an important role in phagocytosis of apoptotic cells as well as myelin and cell debris (Miron and Franklin 2014). Microglia/macrophages have been shown to be both beneficial and detrimental following injury depending on their phenotype (David and Kroner 2011). Classically activated M1 microglia/macrophages are thought to promote tissue damage through their production of pro-inflammatory cytokines (i.e. of IL-1β, TNFα, IL-6, IL-12, IL-23, IFN-γ), proteases, and ROS (Miron and Franklin 2014); whereas alternatively activated M2 microglia/macrophages are associated with phagocytosis of myelin debris and secretion of growth supporting factors (i.e. IL-10, IGF-1) that promote axonal growth and tissue remodelling (Miron and Franklin 2014). Macrophage recruitment is essential following injury as reducing overall inflammation by ablating the macrophage cell pool is associated with worse outcomes (Shechter et al. 2009). Following injury in mice, there is initially a relatively equal numbers of M1 and M2 macrophage/microglia. Over time there is a shift from a relatively equal M1:M2 ratio to an increasingly more prominent M1 inflammatory response and this is associated with inhibition of endogenous repair mechanisms (Kigerl et al. 2009). The microenvironment following SCI has been shown to favour an M1 phenotype (Kroner et al. 2014). Transplantation of M2 macrophages into the spinal cord 7 days post injury results in the majority of these cells to become M1 macrophages 3 days post transplantation (Kigerl et al. 2009). M1 microglia are ultimately detrimental to functional

outcomes after injury and contribute to the secondary injury through multiple mechanisms including: 1) Production of high levels of pro-inflammatory cytokines with lower expression of anti-inflammatory cytokines which further promotes inflammation and can itself promote neuronal death (Miron and Franklin 2014); 2) High expression of inducible nitric oxide synthase (iNOS) which directly induces neuronal death (Kigerl et al. 2009); 3) Release of proteolytic enzymes such as matrix metalloproteinases (MMPs) which can deteriorate the blood spinal barrier and promote cell death by degrading cellular integrity (Hansen et al. 2013; Zhang et al. 2011); 4) Induce astrocyte activation and glial scar formation (Liu and Shubayev 2011); 5) Promote axonal dieback and damage to oligodendrocytes (Busch et al. 2009; Horn et al. 2008; David and Kroner 2011; Stirling et al. 2004; Yune et al. 2003; Miron and Franklin 2014). More recent studies have demonstrated that modulating the inflammatory response to an M2 phenotype promotes functional outcomes in multiple injury models including MS and SCI (Miron and Franklin 2014). The mechanisms behind these benefits are largely unknown but are hypothesized to be due to the release of trophic factors to neighbouring cells as well as increased removal of myelin and cellular debris after injury (Miron and Franklin 2014).

1.3.5.4 B- and T-lymphocytes

B- and T-lymphocyte recruitment peaks around 3 to 7 days post injury but then declines by 50% over the next 2 weeks and remains at that level chronically in rats (Sroga et al. 2003; Jones 2014). Like microglia/macrophages, lymphocytes can either promote tissue damage or repair depending on their activation state. Mice which lack both B- and T-lymphocytes have improvements in their functional recovery after SCI which is associated with monoaminergic axonal growth (Wu et al. 2012). Genetic models in rats that lack T-lymphocytes are associated with improved function and

tissue preservation after SCI (Jones 2014). Similarly, drugs such as cyclosporin A and tacrolimus which block T-cell function are known to promote locomotor recovery after SCI through multiple mechanisms including reduced macrophage activation and improved survival of both motor neurons and oligodendrocytes (LóPez-Vales et al. 2005; Nottingham, Knapp, and Springer 2002; Madsen et al. 1998). In B-cell knockout mice there is improved functional outcome which is associated with increased tissue preservation after SCI (Basso et al. 2006). B-cells are thought to contribute to pathology by promoting microglia/macrophage to remain in a pro-inflammatory M1 state (Ankeny and Popovich 2009; Beck et al. 2010; Peterson and Anderson 2014).

Despite these overall negative associations of B- and T-lymphocytes, there are some beneficial aspects to both cell types as well (Jones 2014). Similar to macrophages, B- and T-lymphocytes represent a diverse lineage of cells with different phenotypes. A specific T-cell subtype, T regulatory cells (T_{regs}), marked by their expression of forkhead box P3 (FOXP3), are known to promote tissue remodelling in the sub-acute phase of injury through their expression of IL-10 (Raposo et al. 2014). Blocking recruitment of T_{regs} following SCI results in increased pro-inflammatory cytokines, tissue destruction, and reduced functional recovery after SCI in mice (Raposo et al. 2014). T_{regs} are also known to induce an anti-inflammatory M2 macrophage phenotype in other injury models such as multiple sclerosis (MS) and Parkinson's disease (Jones 2014). B-cells are less studied after SCI. Findings in other experimental models suggest that a specific subtype of B-cells, B regulatory cells (B10), can promote tissue sparing after stroke (Tedder 2015), however, this has yet to be investigated in SCI.

The various roles both microglia/macrophages and B-and T-lymphocytes play after injury highlights the importance of understanding each cell type's diverse functions (Miron and Franklin

2014). Therapeutics interventions which harness the beneficial roles of immune cells hold the potential to promote functional outcomes after SCI.

1.3.6 Glial Scar Formation

A hallmark of SCI pathophysiology is the activation of astrocytes (i.e. reactive astrogliosis) and the formation of a glial scar (Herrmann et al. 2008; Karimi-Abdolrezaee and Billakanti 2012). The glial scar poses a significant barrier when developing therapies to improve functional recovery, however, its presence is a necessary evil following SCI. Blocking the formation of the glial scar through inhibition of STAT3 has been shown to adversely affect the outcomes of SCI (Okada et al. 2006; Herrmann et al. 2008). Inhibition of reactive astrogliosis is also associated with failure to repair the BBB, leukocyte infiltration, local tissue disruption, severe demyelination, neuronal and oligodendroglial death, and significant decreases in functional recovery (Faulkner et al. 2004; Herrmann et al. 2008; Okada et al. 2006).

Through secondary injury mechanisms activated astrocytes increase their proliferation and migrate to the lesion site to form a protective barrier around the lesion. Reactive astrocytes are phenotypically characterized by hypertrophy, process extension, and increased expression of intermediate filaments nestin, glial fibrillary acidic protein (GFAP) and vimentin (Karimi-Abdolrezaee and Billakanti 2012). Moreover, reactive astrocytes secrete a host of promoting or inhibitory factors with both beneficial and detrimental outcomes on SCI repair and regeneration (Lukovic et al. 2015; Anderson et al. 2016; Karimi-Abdolrezaee and Billakanti 2012; Buffo, Rolando, and Ceruti 2010; Sofroniew 2009; Rideta et al. 1997; Fitch and Silver 2008).

Following SCI, chondroitin sulfate proteoglycans (CSPGs) are dramatically upregulated by reactive astrocytes and are a major component of the glial scar which potently hinders neural

plasticity and regeneration as will be discussed in future sections (Galtrey et al. 2007; Karimi-Abdolrezaee et al. 2010; Barritt et al. 2006a; Bradbury et al. 2002a; Fitch and Silver 2008; Alilain et al. 2011; Grimpe and Silver 2004; Houle et al. 2006). After SCI, several types of CSPGs including neurocan, brevican, versican and NG2 are upregulated, peaking at 2 weeks and persisting chronically (Buss et al. 2009; Asher et al. 2002). Different cell types express various CSPGs and contribute to CSPGs deposition in the ECM matrix following SCI. In future sections, I will discuss the role CSPGs play in both the developing and pathologic CNS and various strategies that are developed to target CSPGs signaling after SCI.

1.3.7 Axonal Degeneration: Die-back and Wallerian Degeneration

Axonal degeneration is an important component of the secondary injury process that occurs after SCI (Lingor et al. 2012). Following SCI, axons which have been cut attempt to regrow and sprout across the injury site (Lingor et al. 2012). This phenomenon is known as axonal sprouting or regeneration. Axons initially form a growth cone, but unfortunately, due to the decreased intrinsic regenerative ability of adult neurons and the inhibitory microenvironment after injury, including the formation of a glial scar containing CSPGs, the majority of these axons fail to regrow or sprout (Dyck and Karimi-Abdolrezaee 2015). After injury, multiple axon terminals can be seen around the injury site in a 'dystrophic state', a hallmark of regenerative failure (Busch et al. 2009; Horn et al. 2008). Most of these dystrophic axons remain stuck in this state and begin a process known as axonal degeneration (Lingor et al. 2012). Axonal degeneration can be divided into axonal die-back and Wallerian degeneration (Lingor et al. 2012). Axonal die-back, or retrograde degeneration, is defined as the retraction of the axon tip away from the initial site of injury after its severance (Lingor et al. 2012). Following spinal cord transection in rat, axons can die-back as

much as 2.5mm from the injury site (Seif, Nomura, and Tator 2007). This die-back has been attributed to infiltrating macrophages which phagocytose dystrophic axon (Busch et al. 2009; Horn et al. 2008). Wallerian degeneration, or anterograde degeneration, also occurs following SCI and is defined as loss of the distal stump of an axon which is separated from the neuronal cell body (Buss et al. 2005; Buss and Schwab 2003). Wallerian degeneration is an inevitable consequence after SCI that also contributes to oligodendrocyte cell death due to disruption in trophic support from degenerating axons (Buss et al. 2005; Buss and Schwab 2003; Alizadeh, Dyck, and Karimi-Abdolrezaee 2015; Abe et al. 1999).

1.3.8 Demyelination

Oligodendrocyte cell loss and demyelination is a critical component of the secondary injury process that occurs following SCI (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Demyelination is damage or loss of the myelin sheath around axons (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). In the chronically injured spinal cord, there is varying degree of demyelination and dysmyelination in the subpial rim surrounding the lesion site (Nashmi and Fehlings 2001). Axons and oligodendrocytes are initially lost through necrosis. As the injury evolves, progressive oligodendrocyte cell loss occurs through apoptosis and autophagy which results in further demyelination of spared axons (Plemel et al. 2014a; McTigue and Tripathi 2008; Abe et al. 1999; Casha, Yu, and Fehlings 2001; Kanno et al. 2009). Spontaneous remyelination occurs after injury by both oligodendrocyte precursor cells (OPCs) and neural precursor cells (NPCs) (Hesp et al. 2015; Salgado-Ceballos et al. 1998; Beattie et al. 1997). However, this remyelination attempt is limited and inadequate due to changes to the post-injury environment (Karimi-Abdolrezaee et al. 2012; Barnabe-Heider et al. 2010; Xing et al. 2014; Hesp et al. 2015; Karimi-Abdolrezaee et al.

2010; Dyck and Karimi-Abdolrezaee 2015). Animal models of demyelinating disease such as MS provide invaluable tools to study myelin-axon interactions and understand the pathological effects of demyelination on axonal integrity and function.

1.3.8.1 Normal Molecular Organization of Myelin and Nodes of Ranvier

Myelin is a modified plasma membrane of oligodendrocytes in the CNS, which enwraps an axon in a segmental and spiral fashion (Barres et al. 1993). Myelination affects function and morphology of axons allowing faster signal propagation with reduced energy consumption (Saab, Tzvetanova, and Nave 2013; Stiefel, Torben-Nielsen, and Coggan 2013; Bishop and Levick 1956; Homma, Mizote, and Nakajima 1983; Frankenhaeuser and Schneider 1951). Several proteins in myelin have been identified to play essential roles in axonal maintenance and function including proteolipid protein (PLP), myelin basic protein (MBP), myelin associated glycoprotein (MAG), and CNPase (2,3-cyclic nucleotide 3-phosphodiesterase) (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015).

Myelinated axons show a high degree of structural organization. A myelinated axon can be separated into distinct domains including node of Ranvier, paranode, juxtaparanode, and internode (Ohno et al. 2014; Plemel et al. 2014a; Eftekharpour, Karimi-Abdolrezaee, and Fehlings 2008a). Node of Ranvier is the gap between two adjacent myelin sheaths and contains high concentrations of voltage-dependent Na^+ channels on the axonal membrane (Amor et al. 2014). Electrical impulse cannot flow through the high resistance myelin sheath, but instead flows through the node of Ranvier and depolarize the axonal membrane at each node resulting in saltatory conduction (Ohno et al. 2011). Paranode is the adjacent segment to the node of Ranvier where myelin loops provide an anchor by tethering the myelin to the axonal membrane (Poliak et al. 1999). Paranodal junctions are critical in preventing lateral diffusion of ion channels along the

axons to ensure proper segregation of Na^+ and K^+ channels at discrete domains on axonal membrane (Gard 1995; Peles and Salzer 2000). The juxtaparanode contains delayed-rectifier *voltage-gated* Kv^+ channels and Na^+/K^+ ATPase channels that allow for rapid exchange of axoplasmic Na^+ for extracellular K^+ and restoration of the resting membrane potential (Traka et al. 2003; Poliak et al. 2003).

1.3.8.2 Demyelination and its Pathophysiological Consequences

Demyelination can contribute to secondary injury mechanisms through a multitude of mechanisms (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Demyelinated axons undergo a host of physiological changes including the complete loss of ion channel distribution, increased metabolic requirements, reduced axonal transport, and increased mitochondrial damage/dysfunction (Davis, Lambert, and Bennett 1996; Kaplan et al. 1997; Nashmi 2000; Eftekharpour et al. 2005; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004; Sinha et al. 2006; Eftekharpour et al. 2007; Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). In addition, myelination provides extrinsic trophic signals, which influence the normal maturation, maintenance, and long-term survival of axons (Mekhail, Almazan, and Tabrizian 2012; Mar and Noetzel 2010; White et al. 2009; Castelvetri et al. 2011; Teixeira et al. 2014; Lassmann, Horssen, and Mahad 2012). The combination of all of these changes ultimately causes axonal die-back and degeneration (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Therapeutic strategies aimed at promoting remyelination have demonstrated the potential to promote axonal sparring and limit progressive axonal dieback in chronic SCI (Karimi-Abdolrezaee et al. 2010). Therefore, our group and others have aimed to promote remyelination in a hope to limit the damage that can occur to an axon while it is

demyelinated. In future sections, I will discuss the therapeutic strategies that have been employed to promote remyelination after SCI.

1.4 Repairing the Injured Spinal Cord

Repair of the injured spinal cord is challenged by environmental restrictions following SCI that ultimately lead to limited functional recovery. Over the past years, several therapeutic strategies have been developed in experimental models to recondition the impermissible environment of injury and promote the outcomes of SCI. In the following sections, I will discuss the limitations present following injury and provide examples of the emerging neuroprotective and neuroregenerative strategies.

1.4.1 Neuroprotective strategies after SCI

Neuroprotective strategies are a popular avenue of research in the SCI field. The goal of these strategies is to limit the initial damage and cellular loss that occurs in the acute stages of SCI including oxidative damage, lipid peroxidation, ionic imbalance, glutamate excitotoxicity and cell death (Wilson, Forgione, and Fehlings 2013). Neuroprotective therapies require acute interventions and therefore are not always therapeutically relevant given that many SCI victims have other clinical issues that need to be addressed first and they often lose the window of opportunity for acute therapies (Fehlings, Wilson, and O'Higgins 2012).

To date, the only approved treatment for acute SCI is methylprednisolone, a high-dose steroid (Hugenholtz 2003); however, debate over the efficacy of this treatment has been raised over the past decade. Animal studies first demonstrated a neuroprotective role of methylprednisolone by limiting lipid peroxidation, calcium influx, and by modulating the

inflammatory response (Braughler and Hall 1984). These preclinical studies led to the first clinical trial for methylprednisolone (National Acute Spinal Cord Injury Studies, NASCIS). In NASCIS II, when administered within 8 hours after injury for a 24 hour time point, high-dose methylprednisolone provided small improvements in both motor and sensory outcomes (Bracken et al. 1990; Bracken et al. 1992). Although promising, subsequent clinical trials did not verify these findings (Bracken et al. 1997). In NASCIS III, acute (8 hour or earlier) infusion of high-dose methylprednisolone for 48 hours had no significant change in motor or sensory outcomes (Bracken et al. 1997). Methylprednisolone treatment also runs an additional risk of incidence of sepsis, pneumonia, hyperglycemia, and gastrointestinal complication and therefore has faced scrutiny among the medical field (Hugenholtz 2003). Despite these confounding results and additional risks, methylprednisolone is commonly used today to treat acute SCI (8 hours or earlier for 24 hours) (Hugenholtz 2003).

Strategies aimed at limiting oxidative damage have been also considered as a vital therapeutic target for SCI. Oxidative damage affects multiple components of the secondary injury cascade as I previously described. Anti-oxidant therapies are an effective strategy to sequester free radicals and limit oxidative damage following injury (Hall 2011; Jana and Pahan 2007; Topsakal, Kilic, et al. 2002; Oyinbo 2011). For example, high-dose vitamin E treatment have shown to improve spinal cord blood flow and reduce lipid peroxidation which is associated with improved hind-limb motor function (Hall 2011).

Other strategies include targeting various ion channels (ie. AMPA, NMDA, Na^+ channels) to limit ionic imbalance and glutamate excitotoxic cell death following injury (Wilson and Fehlings 2013; Ferguson et al. 2008; Beattie, Ferguson, and Bresnahan 2010). These strategies improve tissue preservation which is associated with reduced neuronal and oligodendrocyte death

following SCI (Wada et al. 1999; Yu et al. 1999; Gaviria et al. 2000a; Gaviria et al. 2000b; Grossman and Wrathall 2000; Haghighi et al. 2000; Kanellopoulos et al. 2000). Glutamate levels can rise to toxic levels within 15 minutes after experimental rat SCI and therefore treatment needs to occur very acutely post injury (Kwon, Sekhon, and Fehlings 2010). Administration of Riluzole, a Na^+ channel blocker, results in improvements in functional outcomes after SCI by reducing intracellular Na^+ (Nagoshi, Nakashima, and Fehlings 2015). Interestingly, Riluzole is still effective even when given at 1 and 3 hours post injury in rat SCI indicating that delayed treatment may still promote functional outcomes in human SCI (Wu et al. 2013). Phase I clinical trials have been conducted using Riluzole treatment within 12 hours post injury (Grossman et al. 2013). This study observed gains in mean motor score in grade B cervical injured patients (Grossman et al. 2013). Phase IIB/III clinical trials are currently ongoing (Nagoshi, Nakashima, and Fehlings 2015).

In summary, neuroprotective therapies may hold the greatest potential to improve the outcomes of SCI given that they are limiting the acute secondary injury mechanisms and therefore promoting tissue preservation. However, neuroprotective strategies are not always feasible in human SCI considering they require acute interventions for their maximal effects.

1.4.2 Axonal Sprouting and Regeneration

Following injury, axons are cut and begin an attempted reparative process, however, axons fail to regrow and are subject to die-back. As mentioned previously, failure in axonal growth is attributed to a multitude of factors including the presence of inhibitory molecules, depletion of growth promoting factors and a decreased intrinsic regenerative capacity in adult neurons. Here, I will cover the major strategies that have been developed to promote axonal sprouting and regeneration following SCI. Neurons in the peripheral nervous system (PNS) holds a greater regenerative ability

following axotomy than neurons within the CNS. As a result, many studies have compared the differences that are present between the PNS and the CNS to find ways to promote regeneration after SCI (Huebner and Strittmatter 2009). Interestingly, pioneering studies by Aguayo's group demonstrated that mature CNS neurons are capable of regenerating into peripheral nerve grafts (Richardson, McGuinness, and Aguayo 1982; Richardson, Issa, and Aguayo 1984; David and Aguayo 1981). These seminal findings indicated that the microenvironment in the CNS is a contributing factor to the regenerative failure observed following CNS injury (Huebner and Strittmatter 2009). The main inhibitory factors present within the ECM of the CNS include myelin associated inhibitors (MAIs) and CSPGs (Dyck and Karimi-Abdolrezaee 2015; Huebner and Strittmatter 2009; Bradbury et al. 2002a; Domeniconi et al. 2002; Tang et al. 2001; Tang et al. 1997; Filbin 1995; Mukhopadhyay et al. 1994; McKerracher 2002, 2001; Li et al. 1996; McKerracher et al. 1994). The effects of CSPGs on limiting axonal regeneration will be covered in future sections.

Myelin debris and lack of its clearance are thought to contribute substantially to the regenerative failures observed in the injured CNS. Myelin debris is rapidly cleared in the PNS by glial cells whereas its clearance is rather limited after CNS injury (Huebner and Strittmatter 2009). MAIs including Nogo-A, MAG, and oligodendrocyte myelin-glycoprotein (OMgp) limit neurite outgrowth *in vitro* by signaling through Nogo-66 receptor 1 (NgR1) (Chen et al. 2000; McKerracher et al. 1994; Niederöst et al. 2002; Tang et al. 2001). Interestingly, PNS myelin produced by Schwann cells lacks the majority of MAIs except for MAG (Huebner and Strittmatter 2009). Efforts to block MAIs effects on axonal regeneration have shown varying results in the CNS. MAG appears to play a lesser role in limiting axonal regeneration as MAG knockout mice demonstrated no improvement in corticospinal tract (CST) regeneration following SCI (Bartsch et

al. 1995). Nogo-A knockout mice have shown improved functional recovery following SCI, which is associated with increased CST and raphespinal tract growth (Zheng et al. 2003; Simonen et al. 2003). Therapeutically, antibodies designed to block Nogo-A function promote axonal growth and functional outcomes after SCI and stroke in rats (Wiessner et al. 2003; Maier et al. 2009; Li et al. 2004). Targeting MAIs signaling by blocking NgR1 have also shown improvements in both axonal regeneration and functional outcomes after SCI (Li et al. 2004; GrandPre, Li, and Strittmatter 2002; Fournier, GrandPre, and Strittmatter 2001). Taken together, these findings collectively demonstrate that targeting MAIs is a viable therapeutic target for promoting axonal regeneration and functional outcomes following SCI.

In addition to an inhibitory microenvironment, CNS neurons also have a limited intrinsic ability to regenerate due to their decreased ability to express pro-regenerative associated genes compared to PNS neurons (Seijffers, Allchorne, and Woolf 2006). Following peripheral nerve injury, axons are capable of regeneration and this has been associated with an upregulation in pro-regenerative genes including growth-associated protein-43 (GAP-43), activating transcription factor-3 and c-Jun (Bomze et al. 2001; Raivich et al. 2004; Seijffers, Allchorne, and Woolf 2006). Contrary to the PNS, in the CNS, these pro-regenerative genes are not upregulated (Seijffers, Allchorne, and Woolf 2006). One key difference between PNS and CNS neurons is an elevation in cyclic adenosine monophosphate (cAMP) following peripheral lesions (Qiu et al. 2002; Cai et al. 2001; Carmel, Young, and Hart 2015; Hannila and Filbin 2008). cAMP elevation results in activation of protein kinase A (PKA) and ultimately induces cAMP response element binding protein (CREB) mediated transcription of growth-associated genes (Cai et al. 2002; Hannila et al. 2013; Gao et al. 2004). Interestingly, when a conditioning lesion is performed on peripheral axons one week prior to central injury, it allows for an improvement in sensory regeneration within the

spinal cord which was shown to be due to the upregulation of cAMP (Neumann and Woolf 1999; Qiu et al. 2002). Based on these findings, increasing cAMP levels through pharmacological approaches is a potential therapeutic target for SCI (Hannila and Filbin 2008; Martinez and Gil 2014; Nikulina et al. 2004). For example, Rolipram, a phosphodiesterase 4 inhibitor, is known to increase cAMP by blocking its degradation (Nikulina et al. 2004; Costa et al. 2013). Rolipram treatment increases serotonergic axonal regeneration into embryonic spinal tissue grafts (Nikulina et al. 2004). Other groups have tried to raise cAMP levels by administering a membrane permeable form of cAMP, dibutyryl cAMP, which has been shown to promote regeneration of sensory axons (Qiu et al. 2002; Neumann et al. 2002; Martinez and Gil 2014).

Taken together, these data represent some of the challenges researchers currently face developing therapies to promote the axonal regeneration following SCI. Generally speaking, studies which show axonal sprouting and regeneration typically result in improved functional outcomes after injury (Lu et al. 2012; Baptiste and Fehlings 2007). Developing therapies which promote axonal growth following injury is a therefore a targeted therapeutic approach for promoting functional outcomes after SCI in humans.

1.4.3 Cell Replacement and Remyelination Strategies in SCI

Following SCI, neurons and oligodendrocytes undergo substantial loss in their population. Death of oligodendrocytes and failure to replace them leads to demyelination, the loss of myelin sheath surrounding damaged axons, and consequently functional deficits after SCI (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Replacing lost cells, particularly oligodendrocytes, is a realistic therapeutic target to promote functional outcomes following SCI (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a; Keirstead et al. 2005). Mature oligodendrocytes are post-mitotic

and are therefore unable to contribute to cell renewal following SCI (Keirstead and Blakemore 1997). Efforts from our group and others have been made to enhance oligodendrocyte replacement after SCI by transplanting stem or progenitor cells into the injured spinal cord or by activating endogenous progenitor cells (Keirstead et al. 2005; Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Parr et al. 2008; Hofstetter, Holmstrom, et al. 2005). These studies have revealed that the inadequate replacement of oligodendrocytes following injury is mainly attributed to the inhibitory modifications in the post-SCI niche in which will be discussed in following sections.

1.4.3.1 Neural and Oligodendrocyte Precursor Cells for SCI repair: An Overview

The spinal cord contains two populations of cells capable of replacing lost oligodendrocytes; OPCs and NPCs.

OPCs reside throughout the CNS tissue and are characterized by their expression of platelet derived growth factor receptor α (PDGF-α) and neural/glial antigen 2 (NG2) proteoglycan (Levine and Nishiyama 1996; Keirstead, Levine, and Blakemore 1998). They are capable of proliferating and differentiating into mature myelinating oligodendrocytes (Gensert and Goldman 1997). In the normal spinal cord, these cells are responsible for replenishing the oligodendrocyte population (Barnabe-Heider et al. 2010).

NPCs reside along the neuraxis within the ependymal layer of the spinal cord (Weiss, Dunne, et al. 1996), in the lateral ventricles in the subventricular zone (SVZ), and in the subgranaular zone (SGZ) of the dentate gyrus in the hippocampus (Weiss, Reynolds, et al. 1996; Morshead et al. 1994; Vescovi et al. 1993; Kokovay et al. 2010; Reynolds and Weiss 1992a). NPCs are multipotent and can differentiate into all neural cells including neurons, astrocytes and

oligodendrocytes. NPCs residing in the SVZ give rise to neurons which migrate along the rostral migratory stream to the olfactory bulb and are responsible for replenishing the olfactory neuronal cell pool (Kandasamy et al. 2010; Lim, Huang, and Alvarez-Buylla 2007). NPCs derived from the SGZ give rise to granular neurons which migrate to the granule cell layer of the dentate gyrus (Kandasamy et al. 2010; Lim, Huang, and Alvarez-Buylla 2007). In the normal intact spinal cord, NPCs typically only proliferate to maintain their stem cell population (Weiss, Dunne, et al. 1996; Morshead et al. 1994; Reynolds and Weiss 1992b; Meletis et al. 2008). Following injury, NPCs increase their activity but predominantly give rise to only oligodendrocytes and astrocytes within the spinal cord with very few differentiating into neurons (Figure 1.1) (Barnabe-Heider et al. 2010; Karimi-Abdolrezaee et al. 2012; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a). Therefore, it is generally accepted that the environment of the spinal cord promotes gliogenesis. Interestingly, when spinal cord derived NPCs are transplanted into the dentate gyrus these cells differentiate into granule neurons (Kandasamy et al. 2010; Lim, Huang, and Alvarez-Buylla 2007). Conversely, when brain derived NPCs are transplanted into the injured spinal cord; they do not differentiate into neurons despite their intrinsic neurogenic capability in the brain NPC pools (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a). This evidence indicates that the niche in which NPCs are transplanted into dictates their differentiation pattern, and that SCI is a gliogenic environment.

Figure 1.1. NPCs and OPCs reside in the spinal cord and are capable of differentiation into oligodendrocytes
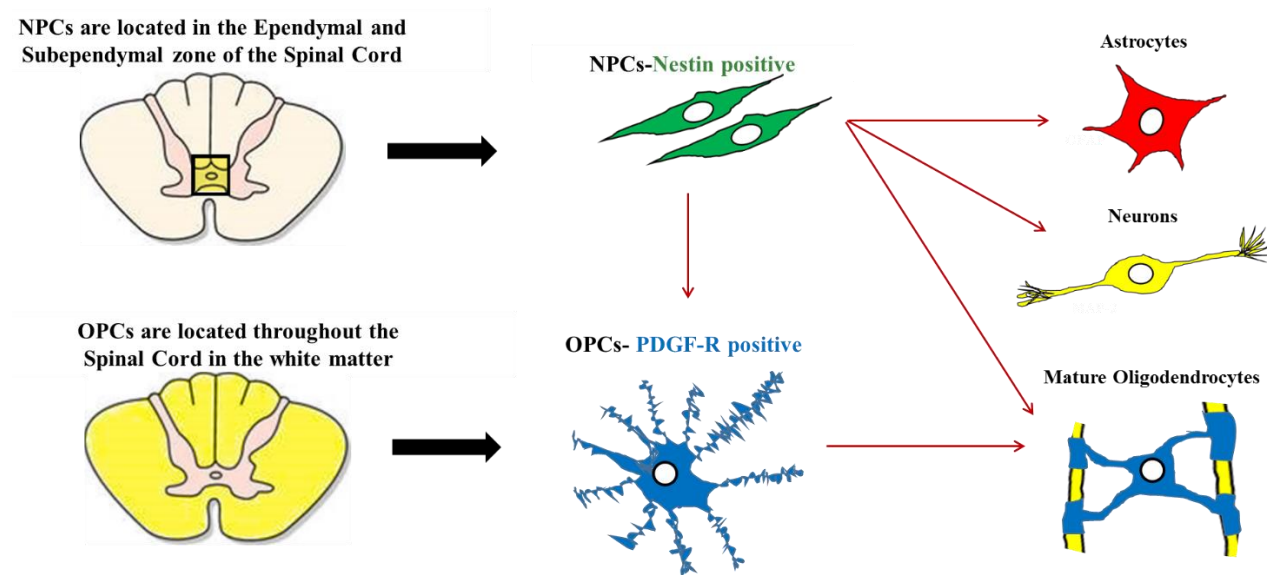
NPCs reside in the ependymal and sub-ependymal zone of the spinal cord (labelled in yellow) and are capable of differentiating into OPCs, astrocytes, neurons and mature oligodendrocytes. OPCs also reside in the spinal cord within the white matter (labelled as yellow) and are capable of differentiating into mature oligodendrocytes.

1.4.3.2 Response of Endogenous Precursor Cells after SCI

Despite the presence of OPCs and NPCs, the adult spinal cord lacks the ability for self-repair (Barnabe-Heider et al. 2010). Following SCI, there is a significant increase in OPCs, NPC, as well as endogenous astrocyte proliferation (Barnabe-Heider et al. 2010; Meletis et al. 2008). OPCs and NPCs contribute to replacing lost oligodendrocytes while NPCs and astrocytes contribute to astrocyte proliferation (Barnabe-Heider et al. 2010; Horner et al. 2000). The number of NG2+ oligodendrocytes surrounding the injury site rises as much as threefold two weeks following injury (McTigue, Wei, and Stokes 2001). Thirty-seven percent of these NG2+ cells co-express PDGFαR indicating the overall rise in the number of OPCs after injury (McTigue, Tripathi, and Wei 2006). By 70 days post injury nearly no NG2+/PDGFαR+ OPCs can be seen in the white matter (McTigue, Tripathi, and Wei 2006). The rise in NG2+/PDGFαR+ cells is thought to be attributed almost entirely to the proliferation of the OPC population after injury as only a limited number of NPCs differentiate to oligodendrocytes and the majority give rise to astrocytes (Barnabe-Heider et al. 2010). Newly formed astrocytes migrate towards the injury epicenter and contribute to the formation of the glial scar (Barnabe-Heider et al. 2010; Sabelström et al. 2013). Interestingly, when the resident NPCs proliferation is restricted after SCI in mice, recovery from injury is poorer than normal, indicating the importance of NPC astrocyte differentiation immediately following injury to limit the initial damage to the spinal cord (Sabelström et al. 2013).

1.4.3.3 Oligodendrocyte Replacement and Spontaneous Remyelination

After SCI, spontaneous remyelination occurs by both OPCs and NPCs, however, the extent and quality of newly formed myelin is limited which results in disorganization of the nodes of Ranvier and continued axonal dysfunction (Nashmi, Jones, and Fehlings 2000; Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004). Immunostainings and electron

micrographs of the injured white matter of the spinal cord demonstrate suboptimal and incomplete remyelination as newly formed myelin is thinner than normally myelinated axons (Nashmi and Fehlings 2001) with aberrant distribution of Kv1.1, Kv1.2 and Caspr (Nashmi, Jones, and Fehlings 2000; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004). Considerable evidence over the past years has uncovered that failure of adequate remyelination is attributed to multiple factors including: 1) the limited replacement of myelinating oligodendrocytes by spinal cord progenitor cells (Karimi-Abdolrezaee et al. 2012; Mothe and Tator 2005a; Barnabe-Heider et al. 2010; Meletis et al. 2008; Gauthier et al. 2013); 2) insufficient levels of key growth factors for oligodendrocyte maturation and myelination (Gauthier et al. 2013; Kakinuma et al. 2004; Almad, Sahinkaya, and McTigue 2011); 3) inadequate clearance of myelin debris that interferes with the process of axonal remyelination (Miron, Kuhlmann, and Antel 2011; Naumann et al. 2003; Lampron et al. 2015); 4) inhibitory factors mainly driven by activated glia such as CSPGs that inhibit migration and maturation of OPCs, differentiation of NPCs to oligodendrocytes, and axonal ensheathment (Karimi-Abdolrezaee et al. 2010; Lukovic et al. 2015; Lau et al. 2012a; Larsen et al. 2003a; Kuhlmann et al. 2008).

Alterations in the expression of essential growth factors for oligodendrocyte development negatively affects the success of oligodendrogenesis and remyelination (Gauthier et al. 2013; Karimi-Abdolrezaee et al. 2006a). Infusion of a cocktail of growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF) and PDGF-AA promotes oligodendrocyte differentiation by endogenous and transplanted NPCs following SCI (Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2012). EGF and FGF co-administration is also known to promote the proliferation of endogenous NPCs, however, in this study no improvements in oligodendrogenesis was observed (Kojima A 2002; Kojima and Tator 2000). The lack of

oligodendrogenesis was likely due to lack of PDGF-AA, which is known to promote the differentiation of oligodendrocytes (Raff et al. 1988). Neuregeulin-1 (Nrg-1) is another important growth factor, which is significantly depleted following SCI (Gauthier et al. 2013). Our groups has shown that restoring the deficient levels of Nrg-1 promotes NPCs proliferation, oligodendrocyte differentiation, and axonal preservation resulting in functional improvement following SCI (Alizadeh 2017; Gauthier et al. 2013).

Oligodendrocyte replacement is also limited by the inhibitory modifications which occur in the post-SCI microenvironment. Presence of myelin debris and insufficient clearance by microglia and macrophages contribute to incomplete remyelination. MAIs are known to inhibit OPCs differentiation and maturation *in vitro* and *in vivo* (Kotter et al. 2006; Plemel et al. 2014a; Nave 2010). Recent *in vitro* studies by Plemel and colleagues indicate that exposure to myelin debris prevents OPCs maturation and their transition to a myelinating phenotype (Plemel et al. 2013). This was demonstrated by a significant decrease in the number of mature oligodendrocytes and was accompanied by increased expression of two proteins, namely inhibitor of differentiation (ID) 2 and ID4, that are known to inhibit oligodendrocyte maturation (Plemel et al. 2013). Thus, proper myelin clearance is an important step required for remyelination to occur (Kotter et al. 2006). In addition to myelin debris, the presence of the dense glial scar, and in particular, CSPGs, is also known to limit oligodendrocyte replacement following SCI (Karimi-Abdolrezaee et al. 2012; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a). The effects of CSPGs on oligodendrocyte replacement and remyelination will be covered in a future dedicated section.

1.4.3.4 Stem Cell Therapies

Over the past decade, efforts from our group and others have been made to enhance oligodendrocyte replacement after SCI by cell transplantation (Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Parr et al. 2008; Hofstetter, Holmstrom, et al. 2005; Gauthier et al. 2013; Keirstead et al. 2005; Sparling et al. 2015). Cell transplantation has shown promising results in enhancing SCI repair through multiple mechanisms including cell replacement, trophic support, immunomodulation and remyelination (Hofstetter, Holmstrom, et al. 2005; Tetzlaff et al. 2011; Vaquero and Zurita 2011; Ogawa et al. 2002; Okano et al. 2003; Rossi et al. 2010; Cummings et al. 2005; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a; Hawryluk et al. 2014; Hawryluk et al. 2012). These studies have suggested that remyelination is a key mechanism in promoting functional recovery in both SCI and demyelination conditions (Yasuda et al. 2011; Sasaki et al. 2006; Eftekharpour et al. 2007; Hawryluk et al. 2014; Karimi-Abdolrezaee et al. 2006a).

The potential of transplanting NPCs or glial progenitor cells to promote remyelination has been explored in a wide variety of pathological conditions such as SCI and MS (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a; Eftekharpour et al. 2007; Cao et al. 2010; Sharp et al. 2010; All et al. 2012; Sun et al. 2013; Hawryluk et al. 2014). These studies have collectively demonstrated the ability of transplanted NPCs to differentiate into myelinating oligodendrocytes and ensheath demyelinated axons. Previous findings from our group in mutant *Shiverer* mice and rat SCI revealed that NPC-derived oligodendrocytes integrate with demyelinated and dysmyelinated axons and successfully remyelinate them (Karimi-Abdolrezaee et al. 2006a; Eftekharpour et al. 2007). When brain-derived NPCs were transplanted into the spinal cord of sub-acutely injured rats, survival and oligodendrocyte differentiation of NPCs was found

to be limited in the injury microenvironment (Karimi-Abdolrezaee et al. 2006a; Eftekharpour et al. 2007). Improving the microenvironment of engrafted NPCs with a cocktail of growth factors (EGF, bFGF, PDGF-AA) considerably promoted their long-term survival, tissue integration and oligodendrocyte differentiation and remyelination (Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010). Importantly, in adult *Shiverer* mice, harboring mutations in MBP and thereby dysmyelination, transplanted NPC myelinated chronically dysmyelinated axons and reconstructed the node of Ranvier (Eftekharpour et al. 2007). In the areas that NPC-derived oligodendrocytes enwrapped and myelinated the *Shiverer* axons, restoration of a normal configuration of paranodal and juxtaparanodal structures was achieved accompanied by improved axonal function in myelinated axons. Similarly, in rat SCI studies, evidence of NPC-derived remyelination was confirmed with immunoelectron microscopy against yellow fluorescent protein (YFP) expression in transplanted YFP-NPCs (Karimi-Abdolrezaee et al. 2006a). Of note, in these studies, transplantation of NPCs also resulted in improved locomotor recovery (Karimi-Abdolrezaee et al. 2006a).

Subsequent studies by Windrem and colleagues in 2008 demonstrated similar outcomes following global transplantation of human glial progenitor cells into the brain of immune-deficient neonatal *Shiverer* mice (Windrem et al. 2008). In this study, transplanted cells successfully differentiated into myelinating oligodendrocyte and functionally myelinated the dysmyelinated host axons in the forebrain and brainstem (Windrem et al. 2008). In agreement with earlier studies, NPCs derived myelination resulted in reconstruction of the node of Ranvier in transplanted neonatal *Shiverer* mice and restoration of transcallosal conduction velocity (Windrem et al. 2008). Moreover, more global myelination in transplanted *Shiverer* mice showed increased lifespan and decreased seizure rate, which is frequently seen in *Shiverer* mice (Windrem et al. 2008).

Collectively, these studies provided proof-of-concept evidence that NPC-derived oligodendrocytes can functionally remyelinate chronically demyelinated axons in SCI and demyelinating lesions.

Recent studies have provided further evidence that implicates remyelination as a key mechanism for neurological improvement observed after transplantation of NPCs in models of SCI (Yasuda et al. 2011; Hawryluk et al. 2014). Yasuda and colleagues transplanted *Shiverer-*derived NPCs that lack the capacity for myelination into the injured spinal cord of NOD/SCID immune-deficient mice (Yasuda et al. 2011). Neuroanatomical, functional, and electrophysiological analyses demonstrated better outcomes in the injured mice transplanted with wild-type NPCs compared to the mice that received *Shiverer* NPCs (Yasuda et al. 2011). This work and similar study by Hawryluk and colleagues (2014) suggest that remyelination is a key mechanism by which NPCs contribute to the functional recovery following transplantation in SCI (Hawryluk et al. 2014).

While NPC transplantation have shown promising results in SCI studies, evidence from our group and others have demonstrated a limited window of opportunity for successful transplantation. These studies have shown that although NPC therapies are beneficial when performed at the sub-acute phase of injury, they ultimately fail in the chronic phase of injury (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a; Keirstead et al. 2005). This failure has been mainly attributed to the maturation of the glial scar and the deposition of CSPGs which will be covered in future sections.

1.5 Chondroitin Sulfate Proteoglycans

Following injury to the CNS, CSPGs expression is markedly upregulated. Injury-induced activation of astrocytes is the main source of increased deposition of CSPGs around the lesion site that results in the formation of a glial scar (Cregg et al. 2014; McKeon, Hoke, and SIlver 1995b; Snow et al. 1990; Rabchevsky et al. 1998; Herrmann et al. 2008; Li et al. 2011). Although initially beneficial in containing the lesion and limiting the spread of inflammatory cells and extent of tissue damage, glial scar formation ultimately inhibits CNS repair in both the sub-acute and chronic stages of injury (for review see Sofroniew 2009; Karimi-Abdolrezaee and Billakanti 2012). CSPGs are considered the main inhibitory components of the glial scar (Grimpe and Silver 2004; Cafferty et al. 2007; Buss et al. 2009) and thereby an attractive target for CNS repair. CSPGs are well studied in the context of SCI; upregulation of CSPGs is well known to limit axonal sprouting (Chen et al. 2002; Barritt et al. 2006a; Massey et al. 2006; Alilain et al. 2011), regeneration (Galtrey and Fawcett 2007; Bradbury et al. 2002a; Fournier, Takizawa, and Strittmatter 2003; Cafferty et al. 2007; Massey et al. 2008; Tom, Sandrow-Feinberg, et al. 2009), conduction (Petrosyan et al. 2013; Arvantan et al. 2009; Hunanyan et al. 2010), as well as replacement of oligodendrocytes and as a result remyelination (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Pendleton et al. 2013; Lau et al. 2012a; Larsen et al. 2003a).

1.5.1 General Overview

CSPGs are a critical component of the ECM. Major components of the ECM in humans include adhesive glycoproteins (e.g., fibronectin, laminin, and tenascin), fibrous proteins (e.g., collagens and elastin), glycosaminoglycans (GAGs) [e.g., hyaluronan, CSPGs, and heparin sulfate proteoglycans (HSPGs)], as well as a wide variety of secreted growth factors and other molecules

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(for review see Galtrey and Fawcett 2007). The ECM of the CNS is unique in that it contains a small amount of fibrous proteins and high amounts of GAGs. Major components include hyaluronan, CSPGs, link proteins, and tenascins (Yamaguchi 2000; Deepa et al. 2006).

CSPGs contain a core protein attached to one or more chondroitin sulfate glycosaminoglycans (CS-GAGs) (Sherman and Back 2008). CSPGs core proteins contribute minimally to their biological activity. The GAGs attached to the core proteins of CSPGs are the main functional component of these proteoglycans (Sherman and Back 2008). Lecticans constitute the majority of the CSPGs in the CNS, including aggrecan, versican, neurocan, and brevican (Yamaguchi 2000). The lecticans share similar structure with N-terminal hyaluronan-binding domains, a central domain that can be glycosylated, and C-terminal globular domains which can bind to other matrix proteins like tenascin R (Yamaguchi 2000). CSPGs NG2 and phosphacan have individual structures, which are unique from the lectican group (Jones et al. 2002). Phosphacan represents the extracellular domain of receptor-type tyrosine phosphatase β (Maurel et al. 1994). Brevican and neurocan are the most abundant CSPGs in the CNS (Yamada et al. 1994; Seidenbecher et al. 1995).

CSPGs GAG carbohydrate chain consists of a long repeating disaccharide units containing D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc) residues which are linked by a β-glycosidic bond (Galtrey and Fawcett 2007). A range of 1 to 100 GAG chains can be attached to one core protein. Epimerization of any of the GlcUA residues to iduronic acid (IdoA) at the polymer level results in the formation of dermatan sulfate (Silbert and Sugumaran 2002). Biosynthesis of CS-GAGs occurs initially in the endoplasmic reticulum with the linkage of xylose by the enzyme xylotransferase to a serine on the core protein (Silbert and Sugumaran 2002; Galtrey and Fawcett 2007). Attachment of two galactose residues to xylose occurs sequentially in the

cis/medial regions of the Golgi by two distinct glycosyl transferases. Formation of the linker section of the GAG chain concludes following the addition of the first GlcUA in the medial/trans Golgi. Chondroitin polymerization occurs sequentially with the addition of GalNAc and GlcUA to the growing side chain by chondroitin synthase. A chondroitin chain can have over 100 repeating disaccharide units (Silbert and Sugumaran 2002; Galtrey and Fawcett 2007). Further modification of these GAG chains occurs through chondroitin sulfotransferase enzymes which add sulfates to the carbohydrates at specific locations (Gama et al. 2006; Akita et al. 2008). The precise sulfation pattern of these polysaccharides defines its protein binding characteristics. These CS-GAGs can be sulfated on carbon (C) 4 of GalNAc (CS-A), C6 of GalNAc (CS-C), C6 of GalNAc and C2 of GlcUA (CS-D), or C4 and C6 of GalNAc (CS-E). Additionally, sulfation of dermatan sulfate (CS-B) occurs on C4 of GalNAc and C2 of GluA. Epimerization at C5 on GluA converts GluA to IdoA (Figure 1) (Gama et al. 2006).

Figure 1.2. Schematic structure of sulfated disaccharides in the chondroitin sulfate chains.
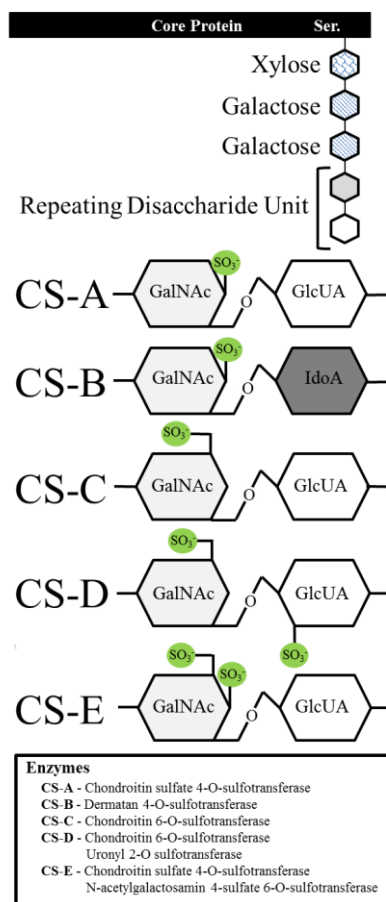
Different sulfation patterns contribute directly to CSPGs complex and diverse functions in both development and in injury. The repeating CS disaccharide units consist of glucuronic acid (GlcUA) (white hexagons) and N-acetylgalactosamine (GalNAc) (light gray hexagons). These CS-disaccharide units are modified by four different sulfotransferases: chondroitin sulfate 4-O-sulfotransferase (CS-A, CS-E), chondroitin 6-O-sulfotransferase (CS-C, CS-D), uronyl 2-O-sulfotransferase (CS-D), and N-acetylgalactosamin 4-sulfate 6-O-sulfotransferase (CS-E). The activity of the chondroitin sulfotransferases leads to the addition of sulfate groups at defined positions (green circles), which results in the generation of specified CS units. GlcUA can be converted to iduronic acid (IdoA) (dark gray hexagons) by C-5 epimerization. The enzyme dermatan 4-O-sulfotransferase (CS-B) preferentially adds a sulfate group at the C4 position of GalNAc, which is adjacent to IdoA.

CSPGs function can vary greatly based on the number and location of CS-GAG chains, and the extent and position of sulfation on these GAG chains (Galtrey and Fawcett 2007; Sherman and Back 2008; Beller et al. 2013). CS-A and CS-C are the predominant sulfation patterns seen in the adult mouse brain (Maeda 2010). CS-D is predominantly expressed in the cerebellum and has very few CS-E subunits. On the other hand, the cerebral cortex contains predominantly CS-E subunit with few CS-D subunits (Maeda 2010). CS-E has been shown to be important in retinal axonal guidance (Gama et al. 2006; Shimbo et al. 2013), however more work is required to elucidate the impact of the sulfation patterns of CSPGs during development and pathology.

1.5.2 CSPGs expression in SCI

CSPGs production seems to be a multifactorial event in CNS injury. Evidence shows that upregulation of CSPGs is partly induced through the activation of transforming growth factor β (TGFβ) signaling after CNS injuries (Susarla et al. 2011; Schachtrup et al. 2010; Jahan and Hannila 2014). Hemorrhage and influx of blood protein fibrinogen into the CNS serves as an early signal for the induction of glial scar formation through TGFβ/SMAD signaling pathways (Schachtrup et al. 2010). Fibrinogen is a carrier of latent TGFβ and induces the phosphorylation of Smad2 and the production of CSPGs in primary cortical mouse astrocytes (Schachtrup et al. 2010). TGFβ1 in particular has been shown to induce reactivity and proliferation of astrocytes *in vitro* (Johns et al. 1992). Upon binding its receptor, TGFβ type II receptor protein co-localizes with TGFβ type I receptor resulting in subsequent phosphorylation of its intracellular downstream effectors, SMAD proteins, which seem to induce the upregulation of CSPGs (Schiller, Javelaud, and Mauviel 2004; Wang et al. 2006; Susarla et al. 2011; Schachtrup et al. 2010). Phosphorylation of SMAD2/3 results in activation of SMAD4 and its translocation to the nucleus (Dernych and Zhang 2003).

Decreased expression in neurocan was shown following the downregulation of both SMAD2 and SMAD3 in mouse primary astrocyte cultures. Interestingly, knockdowns of SMAD2 and SMAD3 had no effect on brevican expression and phosphacan expression only required SMAD2 (Susarla et al. 2011). Contrary to these findings, a recent study has shown that TGFβ promotes the upregulation of CSPGs through a SMAD independent pathway (Jahan and Hannila 2014). TGFβ administration to primary postnatal cortical astrocytes cultures induced upregulation of aggrecan, brevican and neurocan, which was mediated through a non-SMAD signaling pathway as genetic downregulation of SMAD2 and SMAD4 in astrocytes had no effects on TGFβ induced upregulation of CSPGs (Jahan and Hannila 2014). Instead, the authors demonstrated that TGFβ induces upregulation of CSPGs was mediated through the phosphoinositide 3-kinase (PI3K)/Akt and mTOR axis (Jahan and Hannila 2014).

Other factors have been also implicated in CSPGs upregulation following SCI. EGF signaling through EGF receptor 1 induces neurocan and phosphacan synthesis in cultured astrocytes (Smith and Strunz 2005; Asher et al. 2000; Schachtrup et al. 2010).

1.5.3 Role of CSPGs in Axonal Degeneration, Sprouting and Regeneration

In 1990, studies by Silver's group demonstrated for the first time that the astroglial scar limits neurite outgrowth *in vitro* (Rudge and Silver 1990). The same group later demonstrated that upregulation of CSPGs in the glial scar inhibits axon growth *in vitro* (McKeon 1991; Snow et al. 1990). Subsequent *in vitro* studies revealed that CSPGs inhibit neuronal outgrowth by inducing growth cone collapse (McKeon, Hoke, and Silver 1995a; McKeon 1991; Dou and Levine 1994; Monnier et al. 2003; Ughrin, Chen, and Levine 2003). These initial *in vitro* studies identified a negative role for CSPGs in axon growth.

Over the past decades, the impact of CSPGs on axonal regeneration has been investigated extensively in SCI. Lemons and colleagues first demonstrated that CSPGs expression level is increased following SCI (Lemons, Howland, and Anderson 1999). Treatment with chondroitinase ABC (ChABC) was shown to degrade CSPGs in scar tissue following SCI (Lemons, Howland, and Anderson 1999) and improved regeneration of dopaminergic axons in a nigrostriatal tract injury model (Moon et al. 2001). Bradbury and colleagues were the first to show that degradation of CSPGs with ChABC treatment promoted long tract axonal regeneration beyond the lesion site in both sensory and CST axons which resulted in functional recovery in a rodent model of SCI (Bradbury et al. 2002a). Later, upregulation of CSPGs was also confirmed in the astroglial scar in *post mortem* samples of human SCI indicating the clinical relevance of targeting CSPGs (Buss et al. 2009).

Other studies have further verified CSPGs inhibitory effects on neural plasticity showing degradation of CSPGs with ChABC promotes axonal sprouting of the corticospinal (Barritt et al. 2006a; Wang, Ichiyama, et al. 2011; Karimi-Abdolrezaee et al. 2010), serotonergic (Alilain et al. 2011; Barritt et al. 2006a; Karimi-Abdolrezaee et al. 2010), and sensory fibers (Cafferty et al. 2008; Massey et al. 2006; Cafferty et al. 2007) as well as axonal regeneration of corticospinal (Bradbury et al. 2002a), serotonergic (Tom, Sandrow-Feinberg, et al. 2009) and rubrospinal tracts (Yick et al. 2007). In some studies, ChABC-induced structural plasticity was sufficient to improve some functional recovery in rodent (Bradbury et al. 2002a; Caggiano et al. 2005; Yick et al. 2007; Karimi-Abdolrezaee et al. 2010; Tauchi et al. 2012) and in larger mammal studies (Tester and Howland 2008). Furthermore, ChABC treatment was also associated with improved autonomic function showing improved bladder function in both moderate and severe compression injury models (Caggiano et al. 2005; Fouad et al. 2009).

Ipsilateral brainstem injection of ChABC administration immediately following a dorsal column transection between C6-C7 dorsal root entry zones resulted in improved functional collateral sprouting in the cuneate nucleus examined using cholera toxin B tracing (Massey et al. 2006). This was the first study to directly correlate anatomical evidence of sprouting by spinal cord afferents to functional changes after ChABC treatment (Massey et al. 2006). Altogether, these discoveries provide evidence of the therapeutic benefit of targeting CSPGs to promote axonal sprouting and regeneration following SCI.

CSPGs also induce progressive axonal dieback and atrophy following SCI and ChABC treatment has been shown to be attenuate this process (Karimi-Abdolrezaee et al. 2010; Carter, McMahon, and Bradbury 2011; Carter et al. 2008). Following T12 dorsal column injury in mice, intrinsically labeled corticospinal neurons in layer V of the sensorimotor cortex undergo progressive atrophy, with shrinkage of cell bodies but no evidence of cell death at four weeks post injury (Carter et al. 2008). Intrathecal injection of ChABC immediately following dorsal column lesion demonstrated restoration of soma size in injured corticospinal neurons demonstrating the neuroprotective effects of ChABC treatment (Carter et al. 2008). The same group later demonstrated a comparable rescue from lesion-induced atrophy in rubrospinal neurons following intra-cerebroventricular delivery of ChABC at both acute and chronic (8 week) stages of a C5 lateral column injury model (Carter, McMahon, and Bradbury 2011). Karimi-Abdolrezaee and colleagues also showed a role for ChABC in attenuating axonal dieback of CST fibers in a chronic model of compressive SCI, an effect that was maximized when ChABC was combined with NPC transplantation and growth factors administration (Karimi-Abdolrezaee et al. 2010). Additionally, ChABC administration has been shown to modulate macrophage mediated axonal dieback *in vitro* and following SCI (Busch et al. 2009). Neuroprotective effects of ChABC in attenuating the

progressive loss of long-tract neurons and axonal die-back at chronic stage of SCI indicate the long term detrimental effects of CSPGs well after the formation and maturation of the glial scar (Carter, McMahon, and Bradbury 2011; Karimi-Abdolrezaee et al. 2010).

1.5.4 Role of CSPGs in Cell Replacement and Remyelination

Emerging evidence also suggests an inhibitory role for CSPGs in oligodendrocyte differentiation and axonal remyelination after SCI. As indicated earlier, oligodendrocyte replacement and remyelination is a critical repair process in SCI as well as other demyelinating disease such as MS. Activation of endogenous cells or transplantation strategies have been shown to be a feasible therapeutic approach for enhancing remyelination (Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010; Keirstead et al. 2005; Karimi-Abdolrezaee et al. 2012). In demyelinating conditions, OPCs and oligodendrocytes can be found in the vicinity of demyelinated axons, yet many of these cells do not support the formation of compact myelin (Pohl et al. 2011; Wang, Cheng, et al. 2011). In SCI lesions, transplantation of NPCs and OPCs into the injured spinal cord aid in replacing damaged oligodendrocytes and remyelination (Keirstead et al. 2005; Karimi-Abdolrezaee et al. 2006a). Interestingly, in chronic SCI, remyelination was limited after transplantation of human embryonic stem cells (hESC)-derived OPCs (Keirstead et al. 2005) and rodent-derived adult NPCs (Karimi-Abdolrezaee et al. 2006a). In both studies by Keirstead et al and Karimi-Abdolrezaee et al, remyelination occurred after subacute transplantation of OPCs and NPCs transplantation indicating the presence of an inhibitory environment within chronic lesions that prevents axonal ensheathment and/or remyelination (Karimi-Abdolrezaee et al. 2006a; Keirstead et al. 2005). Subsequent studies by Karimi-Abdolrezaee et al. identified that the establishment of the glial scar and deposition of CSPGs in chronic lesions is an underlying cause

of poor survival and oligodendrocyte differentiation of transplanted NPCs following SCI (Karimi-Abdolrezaee et al. 2010). Collectively, these findings suggest that long-lasting deposits of CSPGs in chronic lesions limit the therapeutic windows of opportunity for cell transplantation strategies following SCI.

The inhibitory role of CSPGs in regulating the activity of endogenous precursor cells was substantiated when ChABC administration enhanced endogenous cell proliferation, oligodendrocyte differentiation and remyelination following SCI (Karimi-Abdolrezaee et al. 2012; Siebert, Stelzner, and Osterhout 2011). *In vitro,* CSPGs inhibit OPC adhesion and inhibit their differentiation into mature oligodendrocytes, thereby inhibiting remyelination (Lau et al. 2012a; Larsen et al. 2003a; Siebert and Osterhout 2011a). Aggrecans are shown to specifically inhibits the ability of OPCs to remyelinate DRG neurons *in vitro* (Pendleton et al. 2013). However, this effect was not through the inhibition of OPCs maturation which was observed in other studies (Larsen et al. 2003a; Lau et al. 2012a). Mechanistically, inhibition of Rho-associated kinase (ROCK) and downregulation of CSPGs receptor protein tyrosine phosphatase sigma (PTPσ) significantly reduce the inhibitory properties of aggrecans and promote oligodendrocyte myelination *in vitro* (Pendleton et al. 2013). Collectively, these studies have identified an inhibitory role for CSPGs on modulating OPC integration, migration, maturation and myelination which limits the reparative potential of OPCs for both SCI and MS repair (Lau et al. 2013).

To date, the effects of CSPGs on NPCs, the other cell type capable of replacing lost oligodendrocytes, is largely unexplored. In a hope to develop novel therapeutic targets to promote both endogenous cell replacement following SCI as well as stem cell therapies, the basis of my thesis is to unravel the underlying mechanisms by which CSPGs regulate NPCs.

1.5.5 Immunomodulatory role of CSPGs

Emerging evidence has begun uncovering the role of CSPGs in modulating the immune response following injury. As mentioned earlier, following insult to the CNS macrophages/microglia can acquire an anti-inflammatory (M2) or proinflammatory (M1) phenotype which can have a major impact on the outcome and pathology of SCI. Recent work has shown that large scale CSPGs digestion with the use of lentiviral delivery of ChABC immediately following SCI promotes an M2 neuroprotective macrophage phenotype accompanied by a reduced cavity size and an increase in the preservation of spinal neurons and axons when assessed at 12 weeks post injury (Bartus et al. 2014). CSPGs digestion led to an increase in CD68 positive phagocytic macrophages 3 days following injury and CD206 positive M2 macrophages 2 weeks post injury (Bartus et al. 2014). Further studies by the same group demonstrated that the anti-inflammatory effects of ChABC were mediated though promotion of IL-10 expression (Didangelos et al. 2014). Intrathecal injection of ChABC by lumbar puncture immediately following injury increased the expression of IL-10 while reducing pro-inflammatory IL-12β (Didangelos et al. 2014). Interestingly, ChABC administration into the intact spinal cord also promoted IL-10 expression and was associated with increased monocyte infiltration (Didangelos et al. 2014). Studies have also shown ChABC promotes inflammatory cell infiltration following nucleus pulposus resolution in rabbits (Ishikawa, Nohara, and Miyauti 1999) which suggests CSPGs may modulate the infiltration of macrophages (Bartus et al. 2014; Didangelos et al. 2014). Blocking CSPGs deposition following SCI with xyloside also promotes the infiltration of blood-borne monocytes into the lesion epicenter (Rolls et al. 2008). In this study, CSPGs deposition was shown to be beneficial immediately following injury by promoting macrophages into an anti-inflammatory phenotype (Rolls et al. 2008). Interestingly, blocking the formation of CSPGs with xyloside treatment immediately following SCI limits

functional recovery whereas delayed administration at 2 days post-injury promotes functional recovery (Rolls et al. 2008). This differential effect was attributed to immunoregulatory effects of CSPGs. Inhibiting CSPGs two days following injury drives macrophages/microglia to a M2 type neuroprotective anti-inflammatory state (Rolls et al. 2008). Conversely, when CSPGs formation is disrupted immediately following injury the macrophages/microglia remain in a M1 type inflammatory state (Rolls et al. 2008). When microglia were cultured onto CSPGs substrate they acquired an activated proliferating phenotype in comparison to microglia grown on poly-D-lysine which maintained a rested ramified morphology (Rolls et al. 2008). However, activation of microglia with lipopolysaccharide (LPS) demonstrated that CSPGs promote a noncytotoxic phenotype with reduced expression of TNFα and nitric oxide. This effect was shown to be mediated through CD44 receptor (Rolls et al. 2008). Additionally, presence of CSPGs was shown to induce the expression of IGF-1, a key factor in neuronal survival, in microglia both *in vivo* and *in vitro* (Rolls et al. 2008). Evidence also suggests that the degraded by-product of CSPGs by ChABC (chondroitin sulfate disaccharides, CSPG-DS) may promote a beneficial inflammatory response following injury (Rolls et al. 2004; Rolls et al. 2006; Ebert et al. 2008). Exposure of microglia to CSPG-DS increased their phagocytic capacity without inducing a toxic phenotype in microglia (Ebert et al. 2008; Rolls et al. 2004). Condition media of CSPG-DS activated microglia was not cytotoxic to photoreceptor cells and partially rescued them from IFN-γ induced apoptosis (Ebert et al. 2008). CSPG-DS were also shown to reduce the pathology of experimental autoimmune encephalomyelitis (EAE) by reducing the number of infiltrating T cells (Rolls et al. 2006). The effects of CSPG-DS on T cells was confirmed *in vitro* where CSPG-DS limited T cell motility and decreased their expression of IFN-γ and TNF-α (Rolls et al. 2006). Currently, the role

and mechanisms of CSPGs in modulating immune response after SCI is not fully defined and will be one focus of my thesis.

1.5.6 Targeting CSPGs following SCI

Given the strong evidence which supports CSPGs inhibitory properties following SCI, therapies have been developed to inhibit and degrade CSPGs. As discussed earlier, most studies have examined the role of CSPGs with the use of bacterial enzyme, ChABC, which cleaves the inhibitory GAG chains from CSPGs (Bradbury et al. 2002a). ChABC treatment has been shown to efficiently remove CSPGs *in vivo* in acute, subacute and chronic stages of injury and in different models of SCI (Barritt et al. 2006a; Bradbury et al. 2002a; Cafferty et al. 2008; Caggiano et al. 2005; Carter, McMahon, and Bradbury 2011; Carter et al. 2008; Chau et al. 2003; Fouad et al. 2009; Fouad et al. 2005; Galtrey et al. 2007; García-Alías et al. 2011; Houle et al. 2006; Ikegami, Nakamura, Yamane, Katoh, Okada, Iwanami, Watanabe, Ishii, Kato, Fujita, Takahashi, Okano, et al. 2005; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Massey et al. 2008; Massey et al. 2006; Moon et al. 2001; Tester and Howland 2008; Tom, Kadakia, et al. 2009; Tom, Sandrow-Feinberg, et al. 2009; Wang, Ichiyama, et al. 2011; Yick et al. 2007).

Acute inhibition of CSPGs synthesis has been also tested in multiple studies. Use of a DNA enzyme which targets xylotransferase-1 (XT-1) mRNA, a key enzyme required for glycosylation of the protein backbone of proteoglycans, prevents CSPGs deposition in the glial scar (Grimpe and Silver 2004). Although reduced accumulation of CSPGs in the lesion penumbra was achieved, reduction of CSPGs deposition at the lesion center was incomplete which may be attributed to the compensatory activity of XT-2 enzyme (Grimpe and Silver 2004). Inhibiting the assembly of GAG chains to CSPGs core protein with the use of xyloside has also been tested (Lau et al. 2012a; Rolls

et al. 2008). This method of inhibition has been shown to decrease the deposition of CSPGs in the lesion penumbra in both SCI and multiple sclerosis (MS) models (Rolls et al. 2008; Lau et al. 2012a). Additionally, acute administration of decorin, a small leucine rich proteoglycan known to inhibit the activity of TGFβ and epidermal growth factor receptor (EGFR) has been shown to remarkably decrease CSPGs accumulation up to 90% following injury (Davies et al. 2004). Of note, these targeted therapies require acute administration as they only prevent CSPGs formation and deposition and do not remove CSPGs which are already present in subacute or chronic stages of injury. NG2 monoclonal antibodies have been also developed to specifically neutralize the inhibitory properties of NG2, a form of CSPGs, in acute and chronic stages of SCI (García-Alías et al. 2011; Petrosyan et al. 2013; Tan et al. 2006; Ughrin, Chen, and Levine 2003).

Figure 1.3. Therapeutic interventions to inhibit CSPGs.
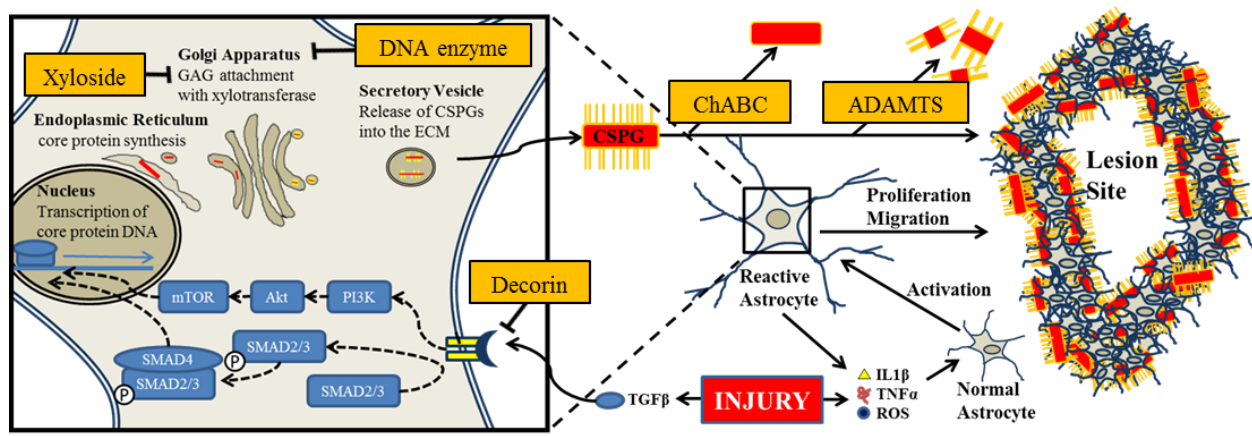
Following injury, an upregulation of TGFβ, IL1β, TNFα, and reactive oxygen species (ROS) induce normal astrocytes to change their function and morphology resulting in reactive astrogliosis. Reactive astrocytes are characterized by increased proliferation, and migration. These reactive astrocytes migrate to the epicenter of the lesion and form a glial scar around the injury site. Reactive astrocytes are known to drastically upregulate inhibitory CSPGs following injury. Evidence has shown that CSPGs upregulation is primarily induced through TGFβ signaling. The downstream signaling cascade that follows TGFβ signaling is controversial, however, it is thought to be mediated through SMAD signaling and/or the PI3K/Akt/mTOR pathway which increases the transcription of CSPGs mRNA.

*Several approaches have been developed to manipulate CSPGs in spinal cord injury. Decorin, a small leucine rich proteoglycan, has been shown to inhibit TGFβ signaling and CSPGs deposition. Xyloside inhibits attachment of GAGs to CSPGs core protein. The bacterial enzyme, Chondroitinase ABC (ChABC) has been used widely to degrade GAG chains to remove inhibitory properties of CSPGs. Additionally, DNA enzymes have been developed which block xylotransferase-1 (XT-1), an enzyme essential for the biosynthesis of GAGs. Recently, ADAMTS4 (**A **D**isintegrin-like **A**nd **M**etalloproteinase with **T**hrombospondin type 1 **M**otif 4) has been shown to inhibit CSPG effects by degrading their core proteins.*

1.5.7 Signaling Mechanisms of CSPGs

Diversity of CSPGs makes it difficult to determine direct mechanisms of CSPGs function. CSPGs have been shown to interact with laminin, fibronectin and neural cell adhesion molecule (NCAM) (Galtrey and Fawcett 2007). Through these interactions, CSPGs exert some of their inhibitory properties by blocking growth-promoting factors.

Several studies have shown that CSPGs directly inhibit the ability of laminin to bind to its integrin receptors (Orlando et al. 2012; Tan et al. 2011; Zhou et al. 2006; Zuo et al. 1998). Integrin receptors function as both adhesion and signaling molecules and their activation promotes extension of filopodia in navigating growth cone towards the synaptic targets (Lemons and Condic 2008). Aggrecan has been shown to directly inhibit laminin-mediated axon growth by impairing integrin signaling through decreasing phosphorylated focal adhesion kinase (FAK) and Src levels (Orlando et al. 2012; Tan et al. 2011). Inducing integrin activation and signaling with manganese enhanced axon growth from cultured rat sensory neurons and human embryonic stem cell-derived motoneurons on aggrecan substrate (Tan et al. 2011).

Previously, it was assumed that CSPGs detrimental effects following SCI were mainly through blocking of growth promoting substances (Galtrey and Fawcett 2007). The recent discovery of four CSPGs receptors uncovered that CSPGs inhibitory actions are additionally modulated through its signaling receptors and activation of growth-inhibiting pathways. Over the past few years, the following receptors have been identified to directly bind to CSPGs: 1) Class II A receptor protein tyrosine phosphates (PTPs), PTPσ, (Shen et al. 2009a; Fry, Chagnon, Lopez-Vales, et al. 2010; Lang et al. 2015), 2) leukocyte common antigen-related phosphatase receptor, LAR (Fisher et al. 2011), 3) Nogo receptor (NgR) family members, NgR1 and 4) NgR3 (Dickendesher et al. 2012).

1.5.7.1 Receptor protein tyrosine phosphatases

The recent discovery of LAR and PTPσ interaction with CSPGs has uncovered new insights into the mechanisms of CSPGs and these receptors in SCI (Shen et al. 2009a; Fisher et al. 2011). These receptors are composed of intracellular tyrosine phosphatase domains that antagonize tyrosine kinase signaling and are thought to control the intracellular phosphatase activity (Ensslen-Craig and Brady-Kalnay 2004). On their extracellular domain, RPTPs contain three Ig domains and 8-9 fibronectin type III (FNIII) repeats which are common to many cell adhesion molecules (McLean et al. 2002). Intracellularly, RPTPs contain two phosphatase domains, D1 and D2. D1 is considered the active region while D2 control interactions with intracellular proteins (Chagnon, Uetani, and Tremblay 2004). Studies carried out in Drosophila were the first to uncover the involvement of RPTPs in neurogenesis showing that motor neurons lacking LAR were unable to reach their appropriate targets (Krueger, Others, and up 1996).

The extracellular domain of LAR and PTPσ has been shown to interact with members of the Trk family of kinases and molecules involved in synaptogenesis, including Slitrk family members, neuroligin-3, and interleukin 1 receptor related protein (Takahashi et al. 2011; Yim et al. 2013; Woo et al. 2009). Additionally, both PTPσ and LAR interact with HSPGs and CSPGs (Fox and Zinn 2005; Aricescu et al. 2002). The following sections discuss LAR and PTPσ and the recent findings that implicate these receptors in mediating CSPG effects.

1.5.7.2 Protein Tyrosine Phosphatase Sigma

In 2009, PTPσ was first identified as a key functional receptor for CSPGs-mediated inhibition of axon growth in the CNS (Shen et al. 2009a). Subsequent studies also showed the impact of targeting PTPσ signaling on axonal regeneration following SCI (Lang et al. 2015; Fry, Chagnon,

Lopez-Vales, et al. 2010). Shen and colleagues demonstrated co-immunoprecipitation of PTPσ with neurocan (Shen et al. 2009a). GAG chains on CSPGs bind to four positively charged lysine residues in the first Ig-like domain of RPTPσ through an electrostatic interaction (Shen et al. 2009a; Yi et al. 2014). The same electrostatic interaction has also been shown with HSPG-PTPσ (Yi et al. 2014; Aricescu et al. 2002). Given CSPG-PTPσ electrostatic interaction, it was anticipated that PTPσ binds with high affinity to highly sulfated CS-D and CS-E, while showing low affinity to singly sulfated CS-A and CS-C (Dickendesher et al. 2012; Yi et al. 2014). CS-A and CS-C are the predominant GAGs in the uninjured mouse brain (Maeda 2010), which would account for the recent findings showing little to no interaction of PTPσ to CSPGs in the adult mouse brain (Yi et al. 2014).

CSPG-PTPσ interaction appears to be critical in the injured CNS (Fry, Chagnon, Lopez-Vales, et al. 2010; Shen et al. 2009a; Sapeiha et al. 2005). Increased neurite outgrowth was observed in DRG neurons (Shen et al. 2009a) and cerebellar granule neurons (Fry, Chagnon, Lopez-Vales, et al. 2010) derived from PTPσ$^{-/-}$ mice which were grown on CSPGs substrate. This effect was shown to be CSPGs specific as PTPσ deletion did not overcome the inhibition of growth imposed by MAG (Shen et al. 2009a). Improved neuronal regenerative capabilities in PTPσ$^{-/-}$ mice have been shown in SCI as well as optic nerve and peripheral nerve crush models (McLean et al. 2002; Thompson et al. 2003; Sapeiha et al. 2005; Fry, Chagnon, Lopez-Vales, et al. 2010; Shen et al. 2009a; Zhou et al. 2014). Evidence shows that the effects of PTPσ activation on axonal growth are mediated, at least in part, through inhibition of Erk1/2 phosphorylation (Sapeiha et al. 2005; Lang et al. 2015). In SCI models, increased growth of sensory axons into the CSPGs-rich lesion area (Shen et al. 2009a) and improved CST regeneration have been demonstrated in the injured PTPσ$^{-/-}$ mice confirming the impact of PTPσ in CSPGs induced regeneration failure (Fry,

Chagnon, Lopez-Vales, et al. 2010). Interestingly, following SCI in lamprey, reticulospinal neurons have heterogeneous regenerative capabilities (Zhang et al. 2014). 'Bad regenerating' neurons which fail to regenerate following a spinal cord transection were found to express PTPσ, whereas those which regenerated past the glial scar showed decreased expression of PTPσ. Those PTPσ expressing neurons showed caspase activation indicating a role for PTPσ in the retrograde neuronal death following SCI (Zhang et al. 2014). Recent genetic studies in cerebellar granular neurons showed that CSPG-PTPσ axis activates Rho while inactivate Akt, Erk, collapsing response mediator protein 2, (CRMP2), S6 ribosomal protein, and CREB (Ohtake et al. 2016).

Recently a novel synthetic peptide, intracellular sigma peptide, ISP, has been developed that blocks PTPσ function by binding to a highly-conserved 24-amino-acid intracellular wedge domain (Lang et al. 2015). PTPσ was shown to limit axonal growth by converting growth cones into a dystrophic state by tightly stabilizing them within CSPGs substrate (Lang et al. 2015). Both PTPσ and LAR were shown to become concentrated in these dystrophic stabilized growth cones. Degradation of CSPGs with ChABC and ISP was able to convert dystrophic growth cones into a motile state. Following contusive SCI in rats, systemic administration of ISP resulted in an improvement in both bladder and locomotor function which was shown to be through restoration of serotonergic innervation below the level of injury (Lang et al. 2015). Altogether, these findings demonstrate the importance of PTPσ in mediating CSPGs inhibitory effects on axonal regeneration.

Interestingly, PTPσ has been shown to have a dual role in neurite outgrowth. Prior to its known function as a CSPGs receptor, PTPσ was known to bind to HSPGs (Aricescu et al. 2002). HSPG-PTPσ interaction exerts positive effects on axonal guidance by promoting outgrowth and synapse formation (Coles et al. 2011; Aricescu et al. 2002), whereas CSPG-PTPσ interaction is

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growth inhibiting (Coles et al. 2011; Shen et al. 2009a). HSPGs cause PTPσ oligomerization on sensory neurons in culture resulting in PTPσ clustering and uneven distribution of phosphatase activity (Coles et al. 2011). Area with lower levels of phosphatases activity could enhance the extent and duration of a phosphorylated state for proteins stimulating neurite extension. Contrary to HSPGs, CSPGs do not cause PTPσ clustering which allows for more regular distribution of PTPσ across the cell membrane causing an increased phosphatase activity across the cell and inhibiting axonal growth (Coles et al. 2011). Differential interactions of PTPσ with CSPGs and HSPGs are due to differences in the sulfation patterns on the GAG chains attached to both types of proteoglycans. Sulfation patterns of CSPGs are relatively uniform, whereas HSPGs have area of high sulfation and areas with little to no sulfation which may explain the drastic differences between CSPG-PTPσ and HSPG-PTPσ interaction (Coles et al. 2011; Murphy et al. 2004).

1.5.7.3 Leukocyte Common-Antigen Related Receptor

LAR receptor is widely expressed in neurons of the adult brain and spinal cord. LAR is important in regulating synapse formation and maturation (Kaufmann et al. 2002; Clandinin et al. 2001; Hofmeyer and Treisman 2009). LAR binds to CSPGs (Fisher et al. 2011), HSPGs (Fox and Zinn 2005) and homophillically to itself (Yang et al. 2003; Yang et al. 2005). Using co-immunoprecipitation with different types of tissue and binding assays in transfected COS-7 cell, LAR was shown to bind to CSPGs with high affinity in a dose-dependent manner (Fisher et al. 2011). Similar to PTPσ, CSPG-LAR interaction is mediated through binding to the first Ig domain of LAR. This was confirmed through point mutation changes of four lysine residues to alanines (K68, K69, K71, and K72) (Fisher et al. 2011). Mutated constructs of the intracellular D1 and D2 domains of LAR identified a role for D1 domain in mediating CSPG-LAR catalytic activity and

the D2 domain is responsible for promoting CSPG-LAR binding (Fisher et al. 2011). The mechanism through which intracellular D2 mediates LAR and CSPG binding is not clear, however, previous findings suggest PTPs dimerization is important for regulating PTPs phosphatase activity and suggests mutations in the D2 domain of LAR may cause conformational changes in PTP dimers which could potentially modify their ligand-binding properties (Fisher et al. 2011). Genetic studies in cerebellar granule neurons, identified that CSPG-LAR activity is meditated through Rho activation as well as inactivation of Akt, Erk, protein kinase C ζ, cofilin and liver kinase B1, similar mechanisms shown for CSPG- PTPσ activity (Ohtake et al. 2016).

Knockout of LAR or its blockade with <u>intracellular LAR peptide (ILP)</u> in mice resulted in a better outcome after SCI including improved locomotor function, CST regeneration and serotonergic axonal sprouting (Fisher et al. 2011; Xu et al. 2015) (Fisher et al. 2011; Xie et al. 2006). ILP is designed to block LAR activity by bindings to the wedge-shaped helix-loop-helix located near the first catalytic domain (Xie et al. 2006). Following thoracic SCI, mice treated with ILP demonstrated improved locomotor function as well as increased axonal growth of descending serotonergic fibers perilesional as well as caudal regions to the injury epicenter (Fisher et al. 2011). These findings provide novel targets for blocking inhibitory effects of CSPGs and improving outcomes following SCI.

Despite the inhibitory outcome of CSPG-LAR interaction, interestingly, when LAR binds homophilically to itself it is growth promoting (Yang et al. 2003; Yang et al. 2005). These findings may explain why LAR deficient mice have been shown to have impaired regeneration following a sciatic nerve injury or entorhinal cortex injury (Xie et al. 2001; Van der Zee et al. 2003). LAR homophilic binding occurs on the fifth fibronectin (FN) type III domain (Yang et al. 2003). Another peptide has been developed to mimic this binding termed L59 (Yang et al. 2005) or

extracellular LAR peptide (ELP) (Fisher et al. 2011). ELP was shown to promote growth in hippocampal neurons and of DRG explants by activating Src, FAK, and TrkB as well as its downstream signaling intermediates including PKC, Erk, Akt and CREB (Yang et al. 2005). In a separate study, ELP peptide promoted the neurite growth of DRG and cerebellar granular neurons on a CSPGs substrate *in vitro* (Fisher et al. 2011). Similar findings were observed *in vivo* following thoracic SCI in mice. ELP treatment resulted in an increase growth of descending serotonergic fibers associated with improved functional recovery (Fisher et al. 2011).

Altogether, current evidence demonstrates that LAR can be both growth promoting and growth inhibiting depending on the context and its interactions with matrix components. A better understanding of the LAR mechanisms of action in the CNS will allow designing effective strategies to promote axon regeneration following injury.

1.5.7.4 Nogo-66 Receptors

Myelin inhibition of neurite outgrowth is primarily mediated through Nogo receptors (NgRs) (He et al. 2003). Three NgR homologs exist but only NgR1 and NgR2 are known to bind to MAIs as I previously discussed. Recent findings have identified NgR1 and NgR3 as CSPGs receptors. Dickendesher and colleagues demonstrated high affinity binding of CSPGs with these receptors and demonstrated significant regenerative growth in double knockout NgR1/3 mice (Dickendesher et al. 2012). Interestingly, this regenerative growth was not observed with knock out of individual NgR, suggesting NgR may have a limited role in CSPGs signaling. Importantly, a further enhancement of axon regeneration was observed in triple NgR1, NgR3, and PTPσ knockout mice. NgR1, NgR3 and PTPσ all show specificity to monosulfated CS-B and disulfated CS-D and CS-E but not CS-A or CS-C (Dickendesher et al. 2012).

In conclusion, the discovery of CSPGs receptors has opened new avenues for uncovering CSPGs complex inhibitory mechanisms. Importantly, manipulation of these receptors provides new therapeutic targets for overcoming CSPGs inhibitory properties following CNS injury. Emerging evidence is currently shedding light on the potential benefits of inhibiting these receptors for axonal regeneration (Dickendesher et al. 2012; Pendleton et al. 2013; Coles et al. 2011; Fisher et al. 2011; Fry, Chagnon, Lopez-Vales, et al. 2010; Lang et al. 2015). Moreover, inhibition of these receptors in combination with other therapeutic strategies may maximize therapeutic benefits of their inhibition for improving a meaningful functional recovery following SCI.

1.5.7.5 Other Signaling Pathways Involved in CSPGs Function

CSPGs and myelin inhibitors have both been shown to trigger local elevations in calcium at the growth cone. EGFR is thought to mediate this calcium influx (Koprivica et al. 2005). EGFR inhibitor AG1478 was shown to neutralize the neurite outgrowth inhibitory activity of CSPGs and astrocyte ECM *in vitro* (Koprivica et al. 2005; Cua et al. 2013). While it needs further elucidation, manipulation of EGFR may aid in limiting the adverse effects of CSPGs upregulation following SCI.

Recent work from Kuboyama et. al. (2013) has demonstrated the activation of protein kinase A (PKA) signaling is responsible for the stalling of axons grown on CSPGs gradients (Kuboyama et al. 2013). Inhibition of PKA causes the phosphorylation of Paxillin and stimulate the growth of stalled axons on aggrecan gradients (Kuboyama et al. 2013). Expression of a phosphomimetic paxillin was able to overcome CSPGs inhibitory effects and facilitate axonal growth following optic nerve crush *in vivo* (Kuboyama et al. 2013). This finding is surprising given the previous work that has shown elevation of cAMP and its consequential activation of PKA

promotes axonal regeneration following optic nerve injury (Monsul et al. 2004; Kurimoto et al. 2010). Understanding how PKA activity is detrimental for axons growing over CSPGs gradients while is beneficial in allowing axonal growth over other regenerative inhibitors has remained elusive (Batty, Fenrich, and Fouad 2017; Lee, Kalinski, and Twiss 2014; Kuboyama et al. 2013).

CSPG effects require protein synthesis as their inhibitory effects can be reduced through protein synthesis inhibitors (Walker, Ji, and Jaffrey 2012). Axons grown in media containing CSPGs exhibit increased protein translation and reduced growth rates (Walker, Ji, and Jaffrey 2012) which has been correlated with an increase in the levels of RhoA and active RhoA after axonal injury (Dubreuil, Winton, and McKerracher 2003; Conrad et al. 2005). CSPGs have been shown to directly affect the activation of the Rho/Rho-associated kinase (ROCK, a major RhoA effector) pathway through both PTPσ and LAR interaction (Fisher et al. 2011; Ohtake et al. 2016). When active, RhoA promotes F-actin disassembly (Maekawa et al., 1999) in axonal growth cones preventing the recruitment of microtubules required for axonal regeneration and causing growth cone collapse. It is well-established that CSPGs and MAIs Nogo-A, MAG, OMgp (Niederöst et al., 2003) inhibit axonal growth through activation of monomeric GTPase RhoA and ROCK (Lehmann et al. 1999). This convergent inhibitory pathway in the injured CNS provides a potential therapeutic target for CNS regeneration (Yiu and He 2006). Inhibition of the Rho/ROCK pathway limits CSPGs inhibitory effects on neuronal growth *in vitro* (Duffy et al. 2009; Dergham et al. 2002; Monnier et al. 2003). ROCKII^{-/-} DRG neurons are less susceptible to CSPGs inhibitory effect on neurite outgrowth in comparison to wild type DRG neurons (Duffy et al. 2009). Similarly, inhibition of ROCK with Y-27632 promotes DRG neurite outgrowth on CSPGs substrate (Dergham et al. 2002; Duffy et al. 2009). Y-27632 is an ATP-competitive inhibitor of both ROCK-I and ROCK-II and acts by binding to their catalytic site and inhibiting their kinase activity

(Ishizaki et al. 2000). In addition, RhoA inactivation with C3 exoenzyme promotes neurite outgrowth on CSPGs substrate of retinal ganglion cells (Monnier et al. 2003) and cortical neurons (Dergham et al. 2002)

In CNS injuries, blockage of the Rho/ROCK pathway *in vivo* has also shown growth promoting effect on axonal regeneration. Inhibition of the Rho/ROCK pathway with either C3 exoenzyme or Y-27632 promotes axonal regeneration following optic nerve and SCI (Lehmann et al. 1999; Chan et al. 2005; Monnier et al. 2003; Dergham et al. 2002; Fournier, Takizawa, and Strittmatter 2003; Ramer, Borisoff, and Ramer 2004). Interestingly, RhoA inhibition with C3 exoenzyme mitigates cell death following SCI in rodents demonstrating the potential neuroprotective benefits of RhoA inhibition (Dubreuil, Winton, and McKerracher 2003).

Cell penetration with C3 exoenzyme is very low, and as a result has yielded varying results in SCI models (Dergham et al. 2002; Fournier, Takizawa, and Strittmatter 2003; Sung et al. 2003; Dubreuil, Winton, and McKerracher 2003). Ba-210 (trademarked as Cethrin) is a recombinant engineered variant of C3 exoenzyme that can readily cross the dura of the spinal cord and has shown promising effects in rodent SCI models, however, its role in blocking CSPG effects have not been tested (Lord-Fontaine et al. 2008; McKerracher and Anderson 2013). A Phase I/IIa clinical trial of Cethrin has recently come to completion (McKerracher and Anderson 2013). The observed motor recovery in this open-label trial suggests that Cethrin may increase neurological recovery after complete SCI (McKerracher and Anderson 2013). Further clinical trials in SCI patients are planned to establish evidence of efficacy (McKerracher and Anderson 2013).

In conclusion, while the role and mechanisms of CSPGs have been studied in the context of axonal regeneration, their role in regulating endogenous cell differentiation and replacement after SCI is poorly defined. In this thesis I have employed a comprehensive investigation on the

role that CSPGs and their signaling receptors play in NPCs regulation and their ability for oligodendrocyte differentiation using SCI relevant *in vitro* and *in vivo* platforms.

Figure 1.4. Proposed Mechanisms of CSPGs.
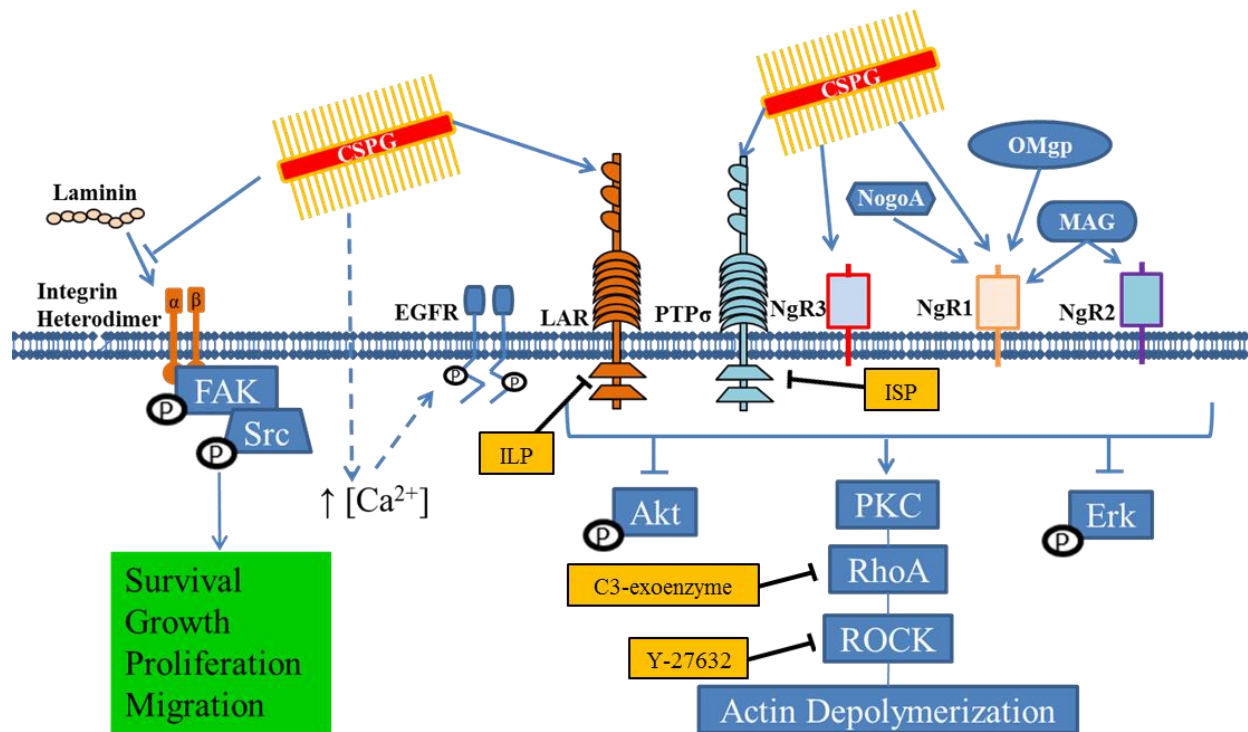
CSPGs exert their effects through multiple mechanisms. Recent evidence has identified four signaling receptors for CSPGs including LAR, PTPσ, NgR1, and NgR3 that mediate the inhibitory functions of CSPGs. Inhibition of LAR and RPTPσ by functionally blocking peptides, ILP and ISP, respectively, has been shown to block CSPG effects. Interestingly, CSPGs also shares a common receptor with myelin inhibitory molecules, NogoA, OMgp, and MAG, present in the injured spinal cord.

At the intracellular level, evidence indicates that CSPGs receptors inhibit Akt and Erk phosphorylation while they activate the Rho/ROCK pathway. Increased activation of the Rho/ROCK pathway results in actin depolymerization and growth cone collapse. Y-27632 and C3-exoenzyme have been utilized to block CSPGs function by blocking the Rho/ROCK pathway. Additionally, CSPGs indirectly inhibit cell growth by limiting the ability of laminin to bind to its integrin receptors. Laminin is a growth promoting extracellular substrate that promote cell survival, growth, proliferation, and migration through FAK and Src phosphorylation. EGFR has been suggested to mediate CSPGs inhibitory function and is thought to be related to calcium influx caused by CSPGs. Inhibition of EGFR activation has been shown to attenuate some of CSPGs inhibitory properties.

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1.6 Thesis Overview

1.6.1 Study Rationale

As reviewed in previous sections, there is a substantial interest in developing cell-based strategies to replace lost neural cells and enhance repair and regeneration after SCI. Adult-derived NPCs have a great potential to replace damaged neural cells various injuries and diseases in the CNS, including SCI (Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010; Keirstead et al. 2005; Hofstetter, Holmstrom, et al. 2005). However, recent studies found that, without interventions, within the environment of SCI endogenous and transplanted NPCs predominantly give rise to astrocytes, and a limited number of oligodendrocytes and neurons (Mothe and Tator 2005a; Hofstetter, Holmstrom, et al. 2005; Karimi-Abdolrezaee et al. 2006a; Karimi-Abdolrezaee et al. 2010; Meletis et al. 2008). Following SCI, increase in the number of astrocytes and changes in their phenotype results in the formation of glial scar around the injury site. The glial scar initially promotes tissue preservation by limiting the spread of inflammation, however, these reactive astrocytes also negatively modify the milieu of spinal cord and limit neural plasticity and axonal regeneration by releasing inhibitory molecules, including CSPGs (Dyck and Karimi-Abdolrezaee 2015). Targeting CSPGs improves axonal growth and functional recovery after SCI (Massey et al. 2006; Bradbury et al. 2002a). Original studies by our group have also shown that degradation of CSPGs with ChABC after SCI promotes the survival and oligodendrocyte differentiation of transplanted (Karimi-Abdolrezaee et al. 2010) and endogenous NPCs (Karimi-Abdolrezaee et al. 2012). The recent discovery of two CSPG specific receptors, PTPσ (Shen et al. 2009a; Fry, Chagnon, Lopez-Vales, et al. 2010) and LAR (Fisher et al. 2011), provides the opportunity to elucidate the role and mechanisms of CSPGs in regulating endogenous cell differentiation and

repair after SCI. Knowledge gained from these studies may also aid in identifying new potential therapeutic targets in overcoming the inhibitory properties of CSPGs following SCI.

1.6.2 General Hypothesis and Research Objectives

The overall goal of my PhD thesis was to elucidate the role and mechanisms by which CSPGs modulate the regenerative response of spinal cord precursor cells after SCI.

Hypotheses:

1. CSPGs negatively influence the ability of NPCs and OPCs for oligodendrocyte differentiation and maturation directly by signaling through LAR and PTPσ receptors.

2. Inhibition of LAR and PTPσ will promote endogenous oligodendrocyte differentiation and preserves mature oligodendrocytes following SCI.

3. Activation of LAR and PTPσ additionally restricts endogenous oligodendrogenesis indirectly by promoting a pro-regenerative immune response after SCI.

Specific research objectives:

1. To investigate the role and molecular mechanisms by which CSPGs regulate the properties of adult spinal cord NPCs *in vitro* (Chapter 2).

2. To evaluate the efficacy of pharmacological inhibition of LAR and PTPσ in overcoming the inhibitory effects of CSPGs on spinal cord derived NPCs using direct *in vitro* assays (Chapter 3).

3. To uncover the role of CSPGs and their signaling receptors LAR and PTPσ in modulating the properties of OPCs *in vitro* (Chapter 3).

4. To determine the therapeutic benefit of blocking LAR and PTPσ signaling on improving the regenerative response of endogenous precursor populations in rat SCI (Chapter 3).

5. To investigate the role of CSPGs and LAR and PTPσ receptors in modulating neuroinflammation using *in vitro* and SCI models (Chapter 4).

These questions have been addressed in three published/under review manuscripts that are reported in Chapters 2-4 of this thesis.

Chapter 2: Chondroitin Sulfate Proteoglycans Negatively Modulate Spinal Cord Neural Precursor Cells by Signaling through LAR and RPTPσ and modulation of the Rho/ROCK Pathway

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Author Contributions: My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 90% of the total experimental procedures, data and analysis. Manuscript preparation was done in collaboration with my supervisor. Neural Stem/Precursor Cell cells were provided by Dr. Santhosh Thomas Kallivalappil. PTPσ knockout mice were created by Dr. Chia-Lun Wu and given to us through a collaboration with Dr. Michel Tremblay's Lab. Immunohistochemical analysis shown in figure 3G-J was done by Dr. Arsalan Alizadeh.

Conflict of Interest: The authors declare no competing financial interests

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2.1 Abstract

Multipotent adult neural precursor cells (NPCs) have tremendous intrinsic potential to repair the damaged spinal cord. However, evidence shows that the regenerative capabilities of endogenous and transplanted NPCs are limited in the microenvironment of spinal cord injury (SCI). We previously demonstrated that injury-induced upregulation of matrix chondroitin sulfate proteoglycans (CSPGs) restricts the survival, migration, integration, and differentiation of NPCs following SCI. CSPGs are long-lasting components of the astroglial scar that is formed around the lesion. Our recent *in vivo* studies demonstrated that removing CSPGs from the SCI environment enhances the potential of adult NPCs for spinal cord repair; however, the mechanisms by which CSPGs regulate NPCs remain unclear. In the present study, using *in vitro* models recapitulating the extracellular matrix of SCI, we investigated the direct role of CSPGs in modulating the properties of adult spinal cord NPCs. We show that CSPGs significantly decrease NPCs growth, attachment, survival, proliferation and oligodendrocytes differentiation. Moreover, using genetic models, we show that CSPGs regulate NPCs by signaling on receptor protein tyrosine phosphate sigma (RPTPσ) and leukocyte common antigen-related phosphatase (LAR). Intracellularly, CSPGs inhibitory effects are mediated through Rho/ROCK pathway and inhibition of Akt and Erk1/2 phosphorylation. Downregulation of RPTPσ and LAR, and blockade of ROCK in NPCs attenuates the inhibitory effects of CSPGS. Our work provide novel evidence uncovering how upregulation of CSPGs challenges the response of NPCs in their post-SCI niche, and identifies new therapeutic targets for enhancing NPC-based therapies for SCI repair.

2.2 Introduction

Spinal cord injury (SCI) results in limited spontaneous tissue regeneration, despite the existence of tissue specific neural precursor cells (NPCs) residing inside the adult spinal cord (Weiss, Dunne, et al. 1996; Johansson et al. 1999). Although showing multipotentiality *in vitro*, recent studies in SCI models demonstrated that without manipulation, NPCs predominantly give rise to astrocytes, and their potential for oligodendrocyte differentiation is restricted following injury (Karimi-Abdolrezaee et al. 2006b; Karimi-Abdolrezaee et al. 2010; Meletis et al. 2008; Barnabé-Heider et al. 2010; Mothe and Tator 2005b; Karimi-Abdolrezaee et al. 2012). Also challenging is the limited migration and long-term survival of NPCs in the injured spinal cord (Karimi-Abdolrezaee et al. 2006b; Karimi-Abdolrezaee et al. 2010). This evidence suggests that the properties of NPCs are negatively modulated by changes in their post-SCI niche. Following injury, extracellular matrix (ECM) undergoes drastic changes mainly driven by activated astrocytes (Karimi-Abdolrezaee and Billakanti 2012; Herrmann et al. 2008; Fitch and Silver 2008). Upregulation of inhibitory chondroitin sulfate proteoglycans (CSPGs) in the post-injury matrix potently impede axon regeneration and plasticity in chronic stages of injury (Lee, Kalinski, and Twiss 2014). Removal of CSPGs with chondroitinase ABC (ChABC) improves axonal regeneration, preservation, sprouting, remyelination, conduction and functional recovery following SCI (Bradbury et al. 2002b; Barritt et al. 2006b; Massey et al. 2006; Karimi-Abdolrezaee et al. 2010; Alilain et al. 2011; Massey et al. 2008; Petrosyan et al. 2013; Arvantan et al. 2009; Hunanyan et al. 2010; Alluin et al. 2014).

We have recently shown that SCI-induced upregulation of CSPGs additionally pose a barrier to cell replacement repair strategies for SCI (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012). Targeting CSPGs by ChABC treatment promoted activities of

endogenous precursor cells and their potential for oligodendrocyte differentiation (Karimi-Abdolrezaee et al. 2012) and optimized the long-term survival and migration of transplanted NPCs in subacute (Ikegami, Nakamura, Yamane, Katoh, Okada, Iwanami, Watanabe, Ishii, Kato, Fujita, Takahashi, Okana, et al. 2005) and chronic (Karimi-Abdolrezaee et al. 2010) stages of SCI. In rat chronic SCI, we demonstrated that ChABC treatment prior to NPCs transplantation resulted in extensive migration of grafted NPCs and allowed their integration with the host tissue suggesting an inhibitory role for CSPGs in modulating the regenerative response of NPCs following injury (Karimi-Abdolrezaee et al. 2010). Currently, the underlying mechanisms of CSPG effects on spinal cord NPCs remain to be identified. Upregulation of CSPGs is a hallmark of CNS injuries including SCI that persists chronically in the NPCs niche (Karimi-Abdolrezaee et al. 2010), thus it is imperative to understand the impact of CSPGs on NPCs activities. The recent discovery in identifying CSPG specific receptors, including protein tyrosine phosphatase receptor sigma, RPTPσ (Shen et al. 2009b) and leukocyte common antigen-related phosphatase, LAR (Fisher et al. 2011), allows us to uncover CSPGs cellular mechanisms.

Here, using comprehensive *in vitro* assays mimicking the matrix of post-injury milieu, we demonstrate that CSPGs directly inhibit NPCs properties including growth, attachment, survival, proliferation, and oligodendrocyte differentiation. CSPGs inhibitory effects are mediated through signaling on both LAR and RPTPσ and intracellularly by the Rho/ROCK pathway. Additionally, CSPGs reduces Akt and Erk1/2 phosphorylation in NPCs which can be overcome by downregulation of LAR and RPTPσ or ROCK inhibition. Our new findings unravel a key role for CSPGs in regulating NPCs and thereby, identify new potential therapeutic options to effectively optimize NPC-based cell replacement strategies for CNS repair.

2.3 Materials and Methods

2.3.1 Animals

All animal procedures were approved by the Animal Ethics Committee of the University of Manitoba in accordance with the policies established by the Canadian Council of Animal Care (CCAC). NPCs were harvested from five C57BL/6, seven BalbC mice (8 weeks of age, Central Animal Facility, University of Manitoba, Canada) and eight RPTPσ-/- mice (12 weeks of age, provided by Michel L. Tremblay, McGill University) (Elchebly et al. 1999). Genotypes of RPTPσ-/- mice were verified using PCR (products of 781 or 1,000 bp). Three adult Sprague Dawley (SD) rats (250g) were used for immunohistological analysis.

2.3.2 Isolation and culturing of adult NPCs

Adult neural precursor cells (NPCs) were isolated from the spinal cord and brain of adult mice (C57BL/6 and BalbC mice) as we described previously (Gauthier et al. 2013; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006b). Briefly, mice were deeply anesthetized by placing in a bell jar saturated with a mixture of 40% isoflurane/60% propylene glycol. Deep anesthesia was confirmed by lack of pedal reflexes. Mice were then killed by decapitation, and the spinal cord was excised under sterile conditions and transferred to artificial cerebrospinal fluid (aCSF) solution. The spinal cord was cleaned of meninges and nerve roots and transferred to 5ml of papain enzymatic solution (Worthington Biochemical Corporation) for 50 min at 37oC. The solution was next replaced by a papain inhibitor mixture and cells were passed through a 70µm cell strainer. The cell suspension was then placed on top of a 7.5% BSA gradient, and centrifuged at 1500 rpm for 7 min. The pellet was resuspended in serum-free medium (SFM, 100 ml) containing 84ml of DMEM/F12 (Invitrogen), 2 ml of 30% glucose, 1.5 ml of 7.5% NaHCO3, 0.5ml of 1M HEPES,

10 mg of transferrin, 2.5 mg of insulin, 0.96 mg of putrescine, 1 µl of selenium, 1 µl of progesterone, 1% L-glutamine, 1% penicillin/streptomycin/neomycin (PSN) and growth factors (1 µg of FGF2 (Fisher, CB40060A), 2 µg of EGF (Sigma, E-4127), and 200 µg of Heparin (Sigma, H-3149). SFM plus growth factors will be referred as growth medium in the text.

2.3.3 Plating NPCs on laminin and CSPGs substrates

Neurospheres were dissociated into single cells and plated onto coated tissue culture surfaces (12,000 cells per cm^2) under different conditions including 1) laminin, 2) laminin+CSPGs, 3) laminin+CSPGs pretreated with chondroitinase ABC (ChABC, Sigma), 4) laminin+ChABC in the media. Prior to cell plating, tissue culture dishes were first coated with Matrigel (50 µl/cm2 of growth surface, 354230, BD Biosciences) for 30 minutes at 37°C. Tissue culture surfaces were washed once with water and then coated with laminin (10ug/mL, Sigma, L2020) and/or CSPG (5ug/mL, Millipore, cc117) for 3 h at room temperature. Of note, CSPGs used in this study contained a mixture of neurocan, phosphocan, versican, and aggrecan. Where appropriate, ChABC (0.1 U/mL Sigma, C3667) was added with laminin + CSPG mixture to tissue culture surfaces for 1 h and incubated at 37°C during coating and prior to cell plating. The cells were grown in NPC growth medium containing EGF and FGF-2 (Sigma) for 2 days assessment or switched to serum medium (SFM plus 2% fetal bovine serum (FBS, Invitrogen) 24 hours following cell plating to induce differentiation (7 days assessments). All *in vitro* assays in this study were conducted in four independent experiments (N=4).

2.3.4 Immunocytochemistry

Dissociated NPCs were plated on laminin and/or CSPG coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) either in growth medium for 2 days assessment or in 2% FBS medium (SFM plus 2% FBS) for 7 days differentiation assay. For proliferation assay, bromodeoxyuridine (BrdU 20 µM, Sigma) was added to the cultures 3 hours before processing NPCs for immunocytochemistry. At the time of immunostaining, cells were fixed with 3% paraformaldehyde (PFA) for 20 minutes at room temperature and washed three times with PBS. Cells were incubated in a blocking solution containing 5% nonfat milk, 1% BSA, and 0.5% Triton X-100 in 0.1 M PBS for 1 hour. This blocking solution was used for all immunostaining procedures unless otherwise has been mentioned. Cultures were then incubated overnight at 4°C with primary antibodies (listed in Table 2.1) diluted in the blocking solution. Cells were washed three times in PBS then incubated with Alexa 568 goat anti-mouse, rabbit, or goat secondary antibody (1:400; Invitrogen) for 1 h. In double staining procedure, the slides were treated with a second primary antibody then incubated with Alexa 488 goat anti-mouse or rabbit secondary antibody (1:400; Invitrogen). The slides were washed three times with PBS and incubated with 4, 6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS) as a nuclear counterstain for 15 minutes. Slides were coverslipped with Mowiol mounting medium. For BrdU immunodetection, prior to blocking, sections were washed with PBS, incubated in 2N HCl and 0.5% Triton X-100 for 30 min at 37oC, and washed with 0.1M sodium borate in PBS for 10 min. After blocking, the slides were then incubated with mouse anti-BrdU overnight and secondary antibodies were added as was previously described. *List of antibodies is provided in Table 2.1.*

Table 2.1. List of antibodies used in this study

Note: ICC: immunocytochemistry, IHC: immunohistochemistry, WB: Western blot

Antibody	Source	Usage	Dilution factor
Actin	Sigma (Rabbit, A2066))	WB	1:300
Actin	Chemicon (Mouse, MAB1501R)	WB	1:300
pan Akt	Cell Signaling (Rabbit, 4691)	WB	1:500
p-Akt	Cell Signaling (Rabbit, 2965)	WB	1:500
BrdU	Santa Cruz (Mouse, 555627)	ICC	1:400
CNPase	Chemicon (Mouse, MAB326R)	WB	1:200
DAPI	Vector (H-1200)	ICC, IHC	1:10000
p44/42 MAPK (Erk1/2)	Cell Signaling (Rabbit, #4695S)	WB	1:1000
P-p44/42 MAPK	Cell Signaling (Rabbit, #4370S)	WB	1:1000
GFAP	Chemicon (Mouse, MAB360)	ICC	1:800
GFAP	Cell Signaling (Mouse, #3670S)	WB	1:5000
Ki67	BD (Mouse, 550609)	ICC	1:50
LAR	Santa Cruz (Rabbit, sc-25434)	ICC IHC	1:50 1:50
LAR	BD (Mouse 610351)	WB	1:250
Nestin	R&D (Goat, AF2736)	ICC	1:500
Nestin	Chemicon (Mouse, MAB353)	ICC	1:500
Olig2	Chemicon (Rabbit, AB9610)	ICC	1:1000
RPTPσ	Santa Cruz (Goat, sc-10871)	IHC	1:50
RPTPσ	R&D (Goat, AF3430)	ICC	1:50
RPTPσ	Novus (Mouse, MAB5861)	WB	1:500
RhoA	Santa Cruz (Mouse, SC-418)	ICC WB	1:50 1:100

2.3.5 Western Blotting

Dissociated NPCs were plated on laminin and/or CSPG coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) either in growth medium for 2 days assessment or in 2% FBS medium for 7 days differentiation assay. For western blotting, cells were harvested from culture plates and homogenized in RIPA buffer (Thermo Fisher) containing SigmaFast Protease Inhibitor (Sigma). A total of 10 to 50 µg of proteins were then loaded on gel and transferred to a nitrocellulose membrane (Bio-Rad). Samples from the same experiment were processed and run on the same gel, and bands were quantified accordingly. The membranes were then blocked in 5% non-fat milk in Tween Tris Buffered Saline (TTBS) and incubated overnight at 4ºC with different antibodies (listed in Table 2.1) diluted in the blocking solution. The membranes were washed and incubated with HRP-conjugated goat anti-mouse or anti-rabbit antibodies (1:4000, Bio-Rad). Membranes were then incubated in ECL plus immunoblotting detection reagents (Thermo Scientific Pierce) according to the manufacturer's specifications. For the RPTPσ antibody, the blocking solution was made of 3% BSA in TTBS. Immunoreactive bands were quantified using AlphaEaseFC (FluorChem, 8900). To control for equal protein loading, membranes were re-probed for Beta-Actin antibody.

2.3.6 Immunohistochemistry on tissue sections

Adult SD rats were deeply anesthetized by placing in a bell jar saturated with a mixture of 40% isoflurane/60% propylene glycol. After deep anesthesia was confirmed by lack of pedal reflexes, mice were perfused transcardially with cold PBS (0.1 M) followed by 2.5% PFA in 0.1 M PBS, pH 7.4. The spinal cords were subsequently dissected and postfixed in the perfusing solution plus 10% sucrose overnight at 4°C. Then, the tissues were cryoprotected in 20% sucrose in PBS for 48

h at 4°C. A 1 cm length of the spinal cord was separated and embedded in tissue-embedding medium (HistoPrep, Fisher Scientific, Pittsburgh, PA) on dry ice. Cryostat sections (35 µm) were cut. For immunostaining, the frozen slides were air dried at room temperature for 30 min and washed with PBS for 5 min. They were then blocked with 1% BSA, 5% non-fat milk, and 0.3% Triton X-100 in PBS for 1 h at room temperature. The primary antibody was applied in the blocking solution overnight at 4°C. A list of the antibodies used in this study can be found in Table 2.1. The slides were washed in PBS three times and incubated with either fluorescent Alexa 488 or 568 goat anti-mouse or anti-rabbit secondary antibodies (1:700; Invitrogen) as appropriate. The slides were washed three times with PBS and coverslipped with Mowiol mounting medium containing DAPI to counterstain the nuclei. The images were taken using a Zeiss 710 laser confocal microscope.

2.3.7 RNA Extraction, Reverse Transcription and Quantitative PCR

NPCs were harvested and homogenized in TRIzol®Reagent (Invitrogen). RNA was then extracted using RNeasy plus mini kit (Qiagen). First-strand cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen). PCR reactions were conducted using the Crimson Taq DNA polymerase (New England Biolabs) in a MyCycler Thermo cycler (Biorad). Primers for LAR and RPTPσ were selected from IDT library. Primers sequences are listed in Table 2.2. Quantitative PCR was performed using an ABI Model 7,700 Sequence DetectorTM (Perkin-Elmer PE Biosystems, USA) using the double stranded DNA specific fluorophore SYBR Green ITM (Invitrogen, Canada). Each SYBR Green reaction (10 µl total volume) contained 4 µl of diluted cDNA (or genomic DNA standard) as template. The final concentrations of the reagents were: 1X SYBR Green PCR Master Mix, 0.1 µM of each primer (50 µM stock). For all the primer sets,

reactions were carried out with our standard SYBR Green protocol with either genomic DNA or cDNA as template as we described previously (Gauthier et al. 2013; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004). ß-actin mRNA was used as internal control. Each real-time quantitative PCR assay was performed twice using triplicate samples. *Sequence of all primers is provided in Table 2.2.*

Table 2.2: PCR Primers used for amplification of LAR, PTPσ, and Actin.

Gene	Primers	Sequence 5'-3'
β-Actin	Forward	5' - CGTGCGTGACATCAAAGAGAA - 3'
	Reverse	5' - GGCCATCTCCTGCTCGAA - 3'
LAR	Forward	5' - CAT CAA ACC AAG CAT CAC GAG – 3'
	Reverse	5' - CGA GAG TGA CTG GTA TAA GCA AG – 3'
RPTPσ	Forward	5' - CTT TGA GTC TCT CCA TGT GCT C – 3'
	Reverse	5' - CTT ACT GAA CAA TGC CGA CCT – 3'

2.3.8 DsiRNA procedures to knockdown RPTPσ and LAR

We used Dicer Substrate interference RNA (DsiRNA) from Integrated DNA Technologies (IDT) to down-regulate RPTPσ and LAR expression in NPCs. NC1 non-target DsiRNA was used as a control. DsiRNA experimental conditions were: 1) NC1, 2) LAR, 3) RPTPσ, 4) LAR + RPTPσ. DsiRNA was reconstituted according to manufacturer's instructions in nuclease-free water and heated at 94°C for 2 minutes and slowly cooled to room temperature. Neurospheres were dissociated and grown in PSN (antibiotic) free growth media at a concentration of 125,000

cells/ml. One day following cell dissociation, NPCs were transfected with DsiRNA solution. Briefly, DsiRNA (10nM) and X-tremeGENE siRNA transfection reagent (5 µl/ml, Roche) were incubated together at room temperature for 20 minutes to allow complexes to form. Transfection complex was added to cells in a drop-wise manner. Transfection efficiency was assessed 24 hours following DsiRNA administration under a fluorescent microscope using positive transfection TYE controls. *Sequence of all DsiRNA is provided in Table 2.3.* Five days following DsiRNA administration, neurospheres were dissociated and harvested for RNA and protein analysis for transfection efficiency or plated onto laminin and/or CSPGs growth substrate.

Table 2.3. List of DsiRNA sequences used.

Note: Uppercase means RNA, lower case means DNA and p is a phosphate

Gene	Strand	Sequence
NC1	S strand	5′ - pCGUUAAUCGCGUAUAAUACGCGUat - 3′
	AS strand	5' - pAUACGCGUAUUAUACGCGAUUAACGAC - 3'
LAR	S strand	5' – pGGCACAAGCAUAACACUGACGCAg – 3'
	AS strand	5' – pCCUGCGUCAGUGUUAUGCUUGUGCCAG – 3'
RPTPσ	S strand	5' – pCCUGCUGUACAAGAACAAACCUGa – 3'
	AS strand	5' – pGUCAGGUUUGUUCUUGUACAGCAGGAU – 3'

2.3.9 Inhibition of Rho/ROCK pathway with Y-27632

ROCK inhibitor Y-27632 was employed in NPC cultures. Neurospheres were dissociated into single cells and pretreated with Y-27632 (10µM) for 1 h before plating on laminin and/or CSPG coated tissue culture.

2.3.10 Assessment of NPCs Attachment

Following 48 hours of NPCs plating on laminin and/or CSPGs, cells were fixed with 3% paraformaldehyde (PFA). Following cell fixation, the slides were washed three times with PBS and incubated with the nuclear marker 4, 6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS) for 15 minutes. Slides were coverslipped with Mowiol mounting medium. Using StereoInvestigator Cavalieri probe, (MBF Bioscience), we randomly measured the total area of cells and their processes in 8-10 separate bright field images (under 20X objective) containing an average of 300 cells for each treatment condition. To estimate the average growth area occupied by each NPC the total area was divided by the total number of DAPI positive cells in each field.

2.3.11 Assessment of NPCs Survival

2.3.11.1 MTT Assay

Dissociated NPCs were plated in 96-well plates at density of 30,000 cells/well either in growth medium for 2 days assessment or in 2% FBS medium for 7 days differentiation. Half of medium was refreshed every three days. For MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, 10 µl of MTT reagent was added to each well and incubated at 37oC for 3 hours. Without removing the media, cells were lysed and purple formazan crystals formed in the mitochondria were solubilized by adding 100ul of 100% DMSO to each well. Absorbance was measured at a wavelength of 570nm with a reference filter at 690nm by a spectrophotometer.

2.3.11.2 LIVE/DEAD Assay

Neurospheres were dissociated into single cells and plated on laminin and/or CSPG coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) in either growth medium for 2 days

assessment or in 2% FBS for 7 days differentiation assay. Half of medium was refreshed every three days. Cell viability was assessed by a LIVE/DEAD assay (Invitrogen). For every 1 ml of PBS, 2 µl of 4 µM ethidium homodimer-1 (EthD-1 assay solution) and 0.5 µl of 2 µM calcein AM assay solution were prepared. To each well, 150 µl of the LIVE/DEAD assay solution was added for 15 minutes at room temperature. The solution was removed and the cultures were washed three times with PBS without shaking. Images of 8-10 randomly chosen fields were taken under 20x objective with a Zeiss fluorescent microscopes with 494nm (green, Calcein) and 528nm (red, EthD—1) excitation filters. For analysis, the percent of live cells was determined by quantifying the total number of LIVE (green) cells compared to the total number of LIVE (green) and DEAD (red) cells in analyzed fields.

2.3.12 Image processing and analysis

For immunocytochemistry quantification, 8-10 separate fields (under 20X objective) containing an average of 300 cells for each condition was randomly imaged (Zeiss AxioObserverZ1 or Imager2 microscope). For each condition, the total number of DAPI positive cells was first assessed, and the number of positive cells for Olig2, GFAP, Ki67 and BrdU (containing a DAPI positive nucleus) were then counted. The percentage of abundance for each cell type was calculated by dividing the number of positive cells for the marker by the total number of DAPI positive cells under each experimental condition. Values were then normalized to control condition for relative comparison.

2.3.13 Statistical Analysis

Data are reported as means ± SEM, and $p \leq 0.05$ was considered significant. Statistical analyses of intensity measurements and cell counts were tested by one-way ANOVA comparing conditions

followed by *post hoc* pairwise multiple-comparison testing by the Holm–Sidak method. For siRNA knockdown experiments, where two conditions were compared, a statistical T-test was used.

2.4 Results

2.4.1 CSPGs negatively modulate the matrix attachment, spreading, survival, and proliferation of spinal cord NPCs

Dissociated primary adult mouse spinal cord NPCs were grown onto substrates containing either laminin or a combination of laminin and CSPGs (laminin + CSPG) for two days (Fig. 2.1A-D). Laminin and CSPGs are highly upregulated in the ECM of the injured spinal cord and therefore laminin+CSPGs condition would more closely represent the milieu of SCI (Buss et al. 2009; Galtrey and Fawcett 2007). We used CSPGs substrate containing a mixture of neurocan, phosphocan, versican, and aggrecan that are present in the ECM of SCI (Galtrey and Fawcett 2007). NPCs exposed to laminin+CSPGs showed a significant decrease in their ability to attach and extend their cell processes (Fig. 2.1B) compared to the NPCs grown on control laminin substrate (Fig 2.1A). Our quantifications showed a 47% decrease in the total number of attached DAPI+ cells in laminin+CSPGs condition (Fig. 2.1F). Moreover, we found a 36% decrease in the total occupied area per NPC on laminin+CSPGs substrate indicating that CSPGs not only limit cell attachment but also cell spreading and growth of the attached NPCs (Fig 2.1E). The specificity of CSPG effects was confirmed by ChABC pre-treatment that is known to remove CSPGs functional properties *in vitro* and *in vivo* (Karimi-Abdolrezaee et al. 2010; Lemons, Howland, and Anderson 1999; Bradbury et al. 2002b; Karimi-Abdolrezaee et al. 2012).

We next assessed whether CSPGs affect the capacity of adult spinal cord NPCs to survive and proliferate. Using an MTT assay, we examined the metabolic activity of NPCs which were

attached to the growth substrate as well as those unattached and floating in the media at 2 days (Fig. 2.1G) and 7 days (Supplemental Fig. 2.1A) post plating. NPCs cultured on laminin+CSPGs substrate demonstrated a significant 38% and 49% decrease in survival at 2 days and 7 days, respectively, in comparison to NPCs plated on laminin alone. Using a complementary LIVE/DEAD assay, we examined the viability of attached cells on the culture substrate (Supplemental Fig. 2.1C-J). NPCs under laminin condition showed 86.31% and 93.45% viability at 2 days (Fig. 2.1H) and 7 days (Supplemental Fig. 2.1B), respectively, whereas NPCs on laminin+CSPGs substrate demonstrated a significant decrease in NPCs survival at 2 days (68.71%). However, we found no difference at 7 days in the percentage of viable attached cells (92.75%) in our LIVE/DEAD assay which may be due to the fact that dead cells may not remain attached to the growth substrate and float in the medium in CSPGs condition.

Ability of NPCs to proliferate following an injury is a prerequisite for their successful regenerative response. Therefore, we examined how CSPGs influence NPCs proliferation using complementary quantitative BrdU incorporation and Ki67 immunostaining assays (Fig. 2.1I-R). Our data revealed a greater number of proliferating BrdU+/DAPI+ cells among NPCs grown on control laminin (43.55%) which was significantly reduced in NPCs plated on laminin+CSPGs (32.73%). Similarly, a significant reduction in the percentage of Ki67+/DAPI+ cells was observed among NPCs grown on laminin+CSPGs substrate (29.19%) in comparison to our laminin control group (42.71%). CSPG effects on survival and proliferation of NPCs were reversed by ChABC confirming specificity of our data. *All values are provided in Supplementary Table 2.1.*

Figure 2.1. CSPGs limit the attachment, growth, survival and proliferation of spinal cord NPCs.
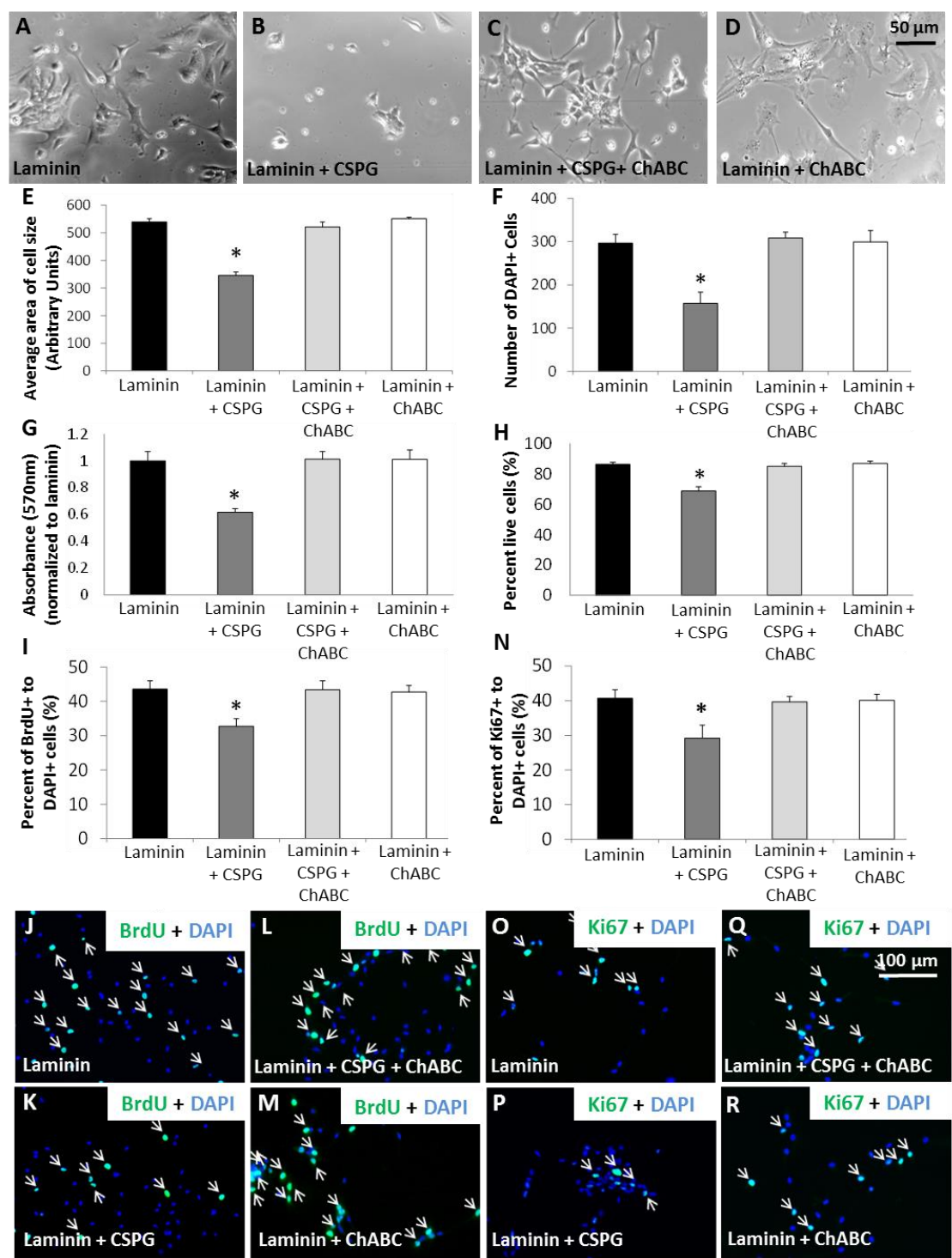
Figure 2.1. CSPGs limit the attachment, growth, survival and proliferation of spinal cord NPCs.

(A-D) Bright field images of spinal cord NPCs grown on laminin (10 µg/ml) or laminin (10 µg/ml) + CSPGs (5 µg/ml) substrate 2 days following cell plating. NPCs grown on laminin+CSPGs substrate (B) showed limited attachment compared to laminin (A). (C) Effects of CSPGs were shown to be specific with ChABC digestion of CSPGs (0.1U/mL) prior to cell plating (E) A significant decrease in the total cell process area of spinal cord NPCs grown on laminin+CSPGs substrate was observed in comparison to all other experimental groups. (F) Additionally, a significant decrease in the number of DAPI positive cells in laminin+CSPGs experimental groups demonstrates that CSPGs limit the attachment of NPCs. (G) Survival of NPCs on CSPGs substrate was assessed using an MTT assay at 2 days following cell plating showing a significant decrease in survival of NPCs grown on CSPGs substrate. (H) Assessment of NPC survival was complimented with a LIVE/DEAD assay using the same experimental parameters 2 days following cell plating. Green cells were labelled as LIVE cells (Calcein) and red as DEAD cells (EthD-1). There was a significant decrease in the percentage of live NPCs when grown on laminin+CSPGs substrate for 2 days. Inhibitory effect of CSPGs on proliferation of spinal cord NPCs was determined by BrdU (I-M) and Ki67 immunostaining (N-R). N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, one-way ANOVA.

2.4.2 CSPGs limit the capacity of spinal cord NPCs for oligodendrocyte differentiation

Following SCI, oligodendrocytes are subject to degeneration and their replacement is essential for axon remyelination (Karimi-Abdolrezaee et al. 2006b; Plemel et al. 2014b). We show that CSPGs drive NPCs to an astrocytic fate and limit their differentiation along an oligodendrocytic lineage. We focused on astrocyte and oligodendrocyte differentiation of spinal NPCs as previous *in vivo* studies by our group and others showed that neuronal differentiation is a rare event in the milieu of spinal cord (Gauthier et al. 2013; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006b; Barnabé-Heider et al. 2010; Meletis et al. 2008). Exposure to CSPGs resulted in a significant 19% increase in the ratio of GFAP positive astrocytes and instead a 47% decrease in the ratio of Olig2 positive oligodendrocytes compared to NPCs were grown on laminin (Fig. 2.2A-E, G-K). Complementary western blotting for GFAP and CNPase (marking mature oligodendrocytes) also verified our immunohistochemical data showing that CSPGs restrict oligodendrocyte differentiation while promoting astrocyte differentiation (Fig. 2.2F, L). ChABC treatment removed CSPGs inhibitory effects on NPCs differentiation. *All values are provided in Supplementary Table 2.1.*

Figure 2.2. CSPGs inhibit oligodendrocyte differentiation in NPCs and drive their fate to astrocytes.
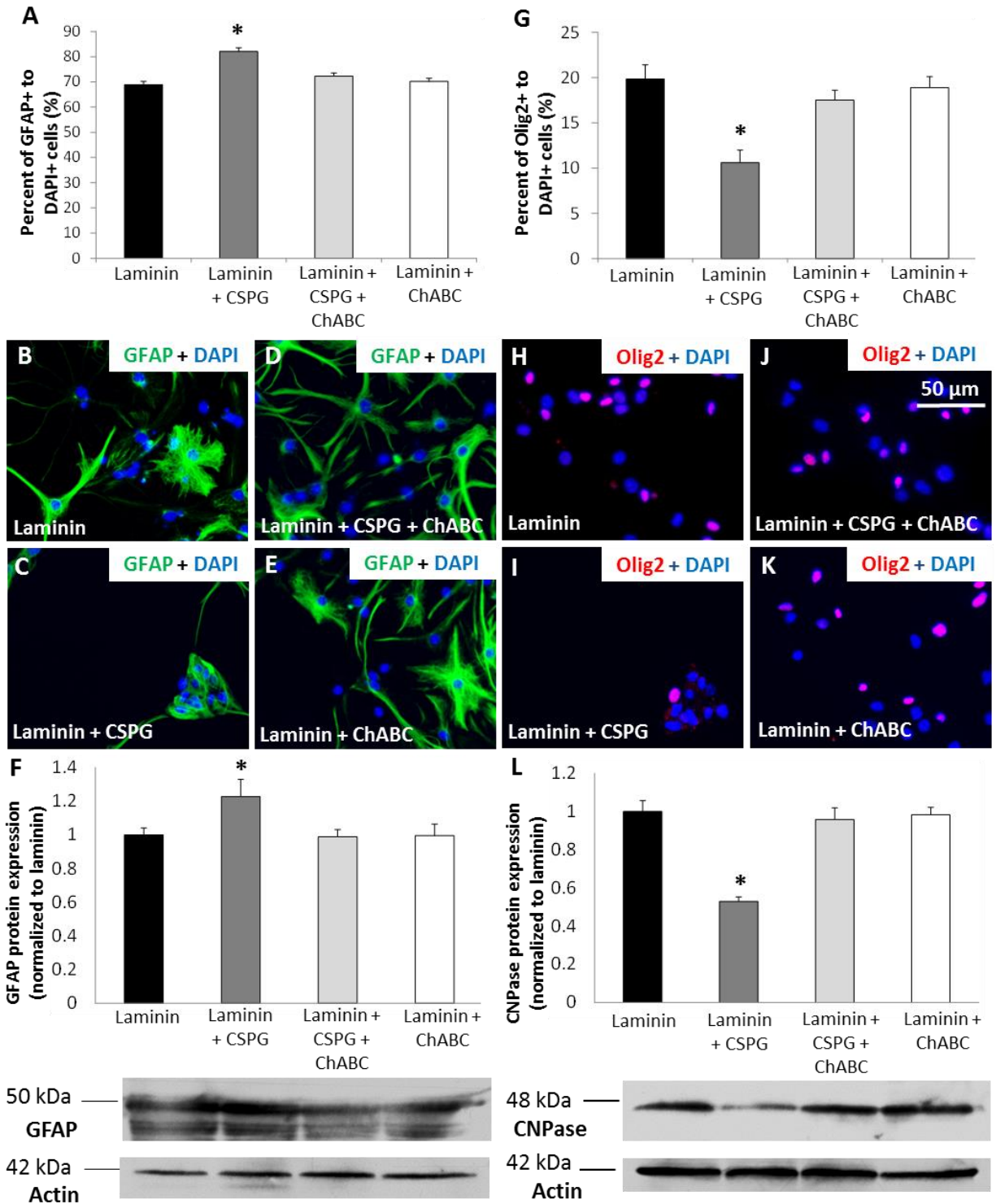
Figure 2.2. CSPGs inhibit oligodendrocyte differentiation in NPCs and drive their fate to astrocytes.

Undifferentiated NPCs were allowed to attach to growth substrates containing laminin (10 μg/ml) or laminin (10 μg/ml) + CSPGs (5 μg/ml). Then they exposed to 2% serum media for 7 days to allow for differentiation. (A) Using quantitative immunostaining on NPCS, we measured the percent of GFAP positive astrocytes to the total number of DAPI positive cells. NPCs grown on laminin+CSPGs substrate (C) showed a significant increase in GFAP+/DAPI+ astrocytes in comparison to NPCs grown on laminin substrate alone (B). Degradation of CSPGs with ChABC (D) removed CSPG effects on astrocyte differentiation suggesting specificity of CSPG effects. (F) Western blotting verified a significant increase in the expression of GFAP protein in NPCs grown on laminin+CSPGs substrate in comparison to all other experimental conditions. (G) Percent of Olig2+/DAPI+ oligodendrocytes was quantified using the same immunostaining assay. NPCs grown on laminin+CSPGs substrate (I) showed a significant decrease in the percent of oligodendrocytes in comparison to laminin only control group (H). (J) Specificity of CSPG effects was confirmed with ChABC pretreatment. (K) ChABC alone had no effects on oligodendrocyte differentiation of NPCs grown on laminin. (L) Complementary Western blotting confirmed CSPG effects on oligodendrocyte differentiation, showing a significant decrease in CNPase protein expression in NPCs grown on laminin+CSPGs substrate compared to control laminin. N = 4 experiments. The data show the mean ± SEM. * P<0.05, one-way ANOVA.

2.4.3 CSPGs modulate the properties of NPCs by signaling through LAR and RPTPσ

We next investigated the cellular mechanisms involved in CSPG effects on NPCs. We focused on two recently identified receptors for CSPGs, LAR and RPTPσ, members of the transmembrane protein tyrosine phosphatase (PTP) receptor family (Shen et al. 2009b; Fisher et al. 2011). Recent studies have implicated both LAR and RPTPσ in mediating the inhibitory effects of CSPGs on axonal growth and regeneration (Fisher et al. 2011; Shen et al. 2009b; Coles et al. 2011; Fry, Chagnon, López-Vales, et al. 2010). Using immunostaining, we first confirmed that spinal cord NPCs express LAR and RPTPσ *in vitro* (Fig. 2.3A-F) and in the ependymal and subependymal region of the rat spinal cord where NPCs reside (Fig. 2.3G-J). We used DsiRNA strategy to downregulate LAR and RPTPσ in NPCs. Using NC1 scrambled DsiRNA as a control, we verified successful downregulation of LAR and RPTPσ transcripts and protein with qPCR and western blotting five days after DsiRNA transfection (Supplemental Fig. 2.2A-E). There was 88% and 71% reduction in LAR mRNA and protein expression, respectively, in NPCs transfected with LAR DsiRNA compared to control NC1 DsiRNA with no changes in RPTPσ expression. Similarly, RPTPσ DsiRNA resulted in 94% and 76% reduction in RPTPσ mRNA and protein levels, respectively, in NPCs treated with RPTPσ DsiRNA compared to NC1 DsiRNA with no changes in LAR expression. In our double knockdown condition of LAR+RPTPσ, successful downregulation of both LAR and RPTPσ mRNA and protein expression was achieved comparable to our single knockdown data. Of note, for each experimental setting that we discuss in following sections we confirmed transfection efficacy and downregulation of LAR and RPTPσ protein. We additionally used NPCs harvested from adult RPTPσ knockout mice to confirm our DsiRNA results. Our characterization of RPTPσ-/- derived NPCs confirmed lack of RPTPσ expression compared to their wildtype counterparts without any effects on LAR expression in NPCs

(Supplemental Fig. 2.3F-H). Moreover, our *in vitro* characterization of RPTPσ-/- NPCs showed that deletion of RPTPσ itself had no apparent changes in the capacity of NPCs for self-renewal, proliferation and differentiation compared to wild-type NPCs (Supplemental Fig. 2.3A-E).

Using these two genetic approaches, we show that downregulation of LAR and RPTPσ remarkably reduces the inhibitory effects of CSPGs on the properties of NPCs. Assessment of the cell attachment and spreading of NPCs (Fig. 2.3K-L, Supplemental Fig. 2.4A-H) showed significant reduction (47.7%) in the number of attached DAPI+ cells in our control NC1 treated NPCs grown on laminin+CSPGs whereas only a 24%, 15%, and 11% reduction was observed in LAR, RPTPσ, and LAR + RPTPσ DsiRNA experimental groups, respectively. Downregulation of LAR and/or RPTPσ also significantly promoted the ability of NPCs to grow and extend their processes on CSPGs substrate. Notably, dual downregulation of LAR and RPTPσ had additive effects on enhancing NPCs attachment and growth on CSPGs compared to their individual knockdown. *All values are provided in Supplementary Table 2.2.*

Knockdown of LAR and RPTPσ receptors promoted survival of NPCs exposed to CSPGs. Complementary MTT and LIVE/DEAD assays confirmed a significant improvement in the survival of NPCs treated with LAR, RPTPσ and LAR + RPTPσ DsiRNA when cells exposed to laminin+CSPGs substrate for 2 days and 7 days (data not shown) compared to our control DsiRNA NC1 condition (Fig. 2.3M, Supplemental Fig. 2.4K). Similarly, BrdU and Ki67 proliferation assays revealed significant improvement in proliferation of NPCs on CSPGs in conditions where LAR and/or RPTPσ were downregulated (Fig. 2.3N, data not shown for Ki67). We found that NPCs treated with LAR, RPTPσ and LAR + RPTPσ DsiRNA showed comparable proliferation capacity compared to NC1 DsiRNA NPCs grown on permissive laminin indicating LAR and RPTPσ have no effect on NPCs proliferation *per se*. Notably, proliferation of NPCs on CSPGs

was statistically increased in RPTPσ and LAR + RPTPσ DsiRNA conditions compared to NC1 DsiRNA group (Fig. 3N) while LAR DsiRNA displayed non-significant increase. Moreover, using NPCs isolated from RPTPσ-/- mice, we confirmed our findings with DsiRNA approach showing significant improvement in the ability of NPCs to attach, grow and proliferate when exposed to CSPGs (Fig. 2/4A-D). *All values are provided in Supplementary Table 2.3.*

By signaling on LAR and RPTPσ, CSPGs regulate the differentiation of NPCs (Fig. 2.3O-P, Supplemental Fig. 2.4I-J). Our complementary immunohistochemical analysis and western blotting using lineage markers for astrocytes (GFAP) and oligodendrocytes (Olig2 and CNPase) revealed that downregulation of both LAR and RPTPσ significantly increased the potential of NPCs for oligodendrocyte differentiation while decreasing astrocyte-derived NPCs when plated on CSPGs in comparison to NPCs treated with control NC1 DsiRNA. The modulatory role of CSPGs/RPTPσ signaling on NPCs differentiation was further confirmed using NPCs from RPTPσ-/- mice in similar experiments (Fig. 2.4E-N). These results indicate that both CSPG/LAR and CSPG/RPTPσ signaling influence NPCs differentiation. Notably, our experiments demonstrated that downregulation of LAR and/or RPTPσ had no apparent effect on the ability of NPCs to attach, grow, and proliferate on laminin indicating the specific role of LAR and RPTPσ in mediating CSPGs functions.

Figure 2.3. LAR and RPTPσ mediate CSPGs inhibitory effects on attachment, growth, survival, proliferation and oligodendrocyte differentiation of NPCs.
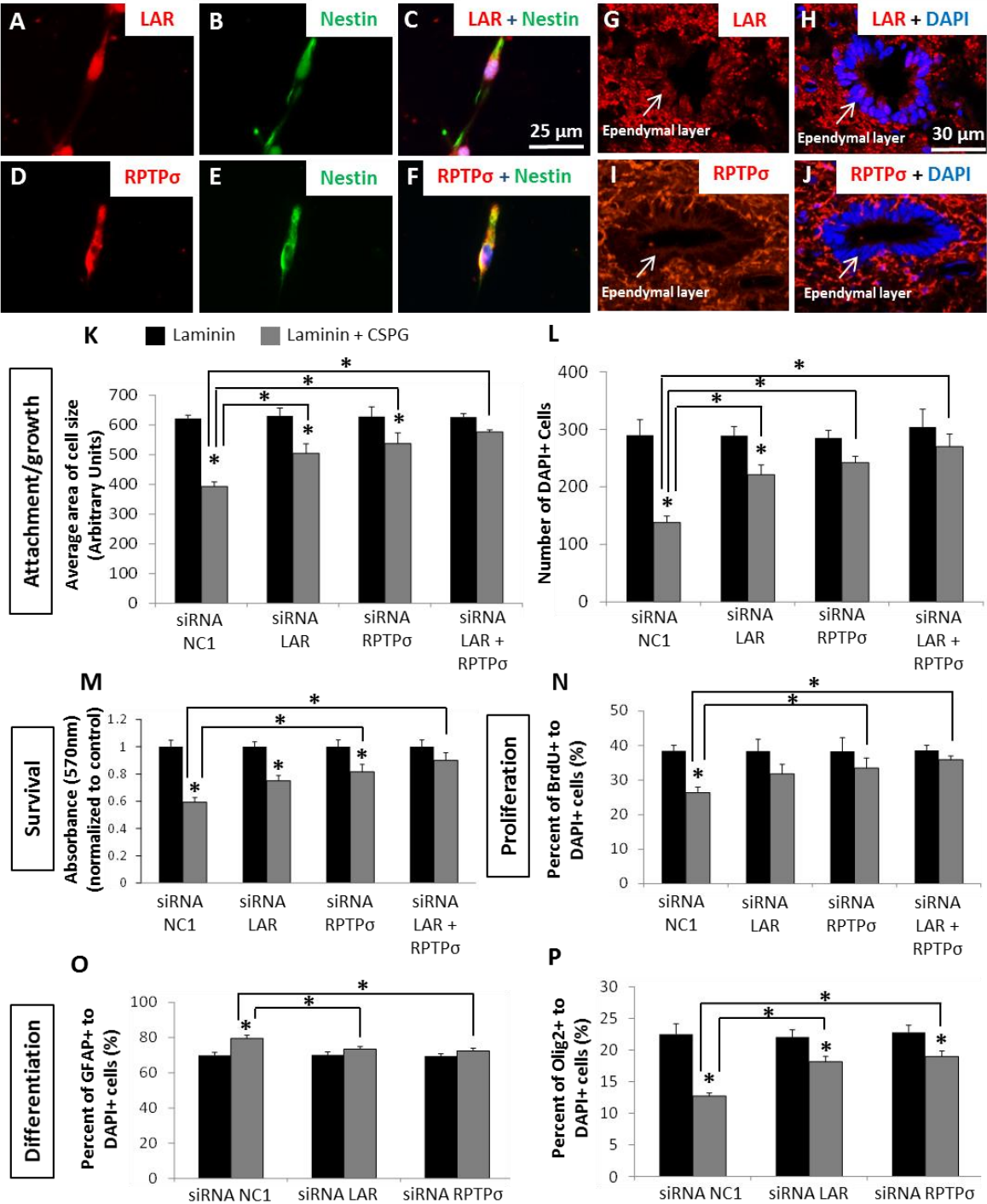
Figure 2.3. LAR and RPTPσ mediate CSPGs inhibitory effects on attachment, growth, survival, proliferation and oligodendrocyte differentiation of NPCs.

In vitro immunostaining verifies the expression of LAR (A-C) and RPTPσ (D-F) by nestin positive NPCs. *In vivo* immunohistochemical analysis of the spinal cord tissue also demonstrated the expression of LAR (G-H) and RPTPσ (I-J) in the ependymal layer of the spinal cord, where NPCs reside. (K-L) Downregulation of LAR and/or RPTPσ significantly increased the ability of NPCs to grow and attach on laminin (10 μg/ml) + CSPGs (5 μg/ml) substrate in comparison to DsiRNA NC1 control condition. (M) Survival of NPCs on CSPGs substrate was assessed using MTT assay 2 days following cell plating. Downregulation of RPTPσ or dual downregulation of LAR + RPTPσ significantly improved the survival of NPCs when grown on CSPGs substrate in comparison to NC1 control group. (N) Downregulation of LAR and/or RPTPσ also promoted the proliferation of NPCs on laminin+CSPGs substrate in comparison to NC1 control; however, this was only statistically significant higher in RPTPσ and LAR + RPTPσ experimental groups. (O-P) Immunostainings of NPCs grown on laminin+CSPGs substrate demonstrate both LAR and RPTPσ mediate CSPGs inhibitory effects on NPC differentiation. Downregulation of LAR or RPTPσ significantly increased the percentage of Olig2 positive cells when grown on laminin+CSPGs substrate in comparison to NC1 control group. In figure M, results from each knockdown experimental condition were normalized to their respective laminin group. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, Student *t* test.

Figure 2.4. RPTPσ knockout NPCs are less susceptible to CSPGs inhibitory effects.

Figure 2.4. RPTPσ knockout NPCs are less susceptible to CSPGs inhibitory effects.
NPCs harvested from wild-type and RPTPσ-/- mice were grown on laminin (10 μg/ml) or laminin
(10 μg/ml) + CSPG (5 μg/ml) substrate in NPCs growth media for 2 days or differentiated in 2%
serum media for 7 days., Lack of RPTPσ in NPCs showed less susceptibility to CSPGs inhibitory
effects on cell spreading (A) and attachment (B) in comparison to the wild type control condition
confirming our DsiRNA findings. Similarly, RPTPσ-/- NPCs were less susceptible to CSPGs
inhibitory effect on survival (C) as was shown through an MTT assay 2 days following cell plating.
(D) RPTPσ-/- NPCs showed a significantly higher percentage of BrdU positive cells in comparison
to wild type NPCs when grown on laminin+CSPGs substrate. (E-N) Using quantitative
immunostaining on NPCs, we measured the percent of GFAP+ astrocytes and Olig2+
oligodendrocytes to the total number of DAPI+ cells under each condition. Wild type NPCs grown
on laminin+CSPGs substrate were preferentially differentiated into astrocytes compared to the
laminin control while RPTPσ-/- NPCs showed lesser degree of astrocyte differentiation compared
to their respective control condition. Conversely, RPTPσ-/- showed a higher degree of
oligodendrocyte differentiation compared to their wild-type counterparts when exposed to CSPGs
demonstrating a role for RPTPσ in mediating CSPGs inhibitory effects on oligodendrocyte
differentiation. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, Student
t test.

2.4.4 CSPG effects on NPCs are mediated through the Rho/ROCK pathway

We next focused on identifying the mechanisms by which CSPGs signaling pathway modulate the properties of NPCs. CSPG activation of the Rho/ROCK pathway has been shown to induce growth cone collapse thereby limiting axonal growth (Wahl et al. 2000; Monnier et al. 2003; Walker, Ji, and Jaffrey 2012). Thus; we asked whether the Rho/ROCK pathway is also involved in exerting the effects of CSPGs on NPCs. Using western blotting, we observed over 2 folds increase in RhoA protein expression in NPCs after exposure to CSPGs (Fig. 2.5A-E). Specificity of CSPG effects on RhoA expression was confirmed by pretreatment of CSPG with ChABC prior to NPC plating. To investigate the functional impact of CSPGs-induced upregulation of RhoA in NPCs, we blocked Rho signaling by inhibition of the downstream ROCK in NPCs with a well-known specific ROCK inhibitor, Y-27632, (Monnier et al. 2003). We treated dissociated adult spinal cord NPCs with 10μM of Y-27632 for 1 hour prior to NPC plating. Y-27632 has been commonly used and shown to efficiently block the ROCK pathway *in vitro* at 10μM concentration (Pendleton et al. 2013; Fournier, Takizawa, and Strittmatter 2003; Walker, Ji, and Jaffrey 2012). ROCK inhibition was able to entirely overcome the inhibitory effects of CSPGs on NPCs growth and attachment (Fig. 2.5F-G, Supplemental Fig. 2.5A-D). Similarly, blockade of the Rho/ROCK pathway reversed CSPGs inhibition of NPC survival and proliferation. Our MTT, LIVE/DEAD, BrdU and Ki67 assays collectively confirmed that pretreatment of NPCs with Y-27632 significantly removed CSPGs inhibitory properties on NPC survival and proliferation restoring it to levels near that of our control laminin group at 2 days following cell plating (Fig. 2.5H-I, data not shown for Ki67, Supplemental Fig. 2.5G).

Additionally, our findings revealed that interruption of the Rho/ROCK signaling in NPCs augmented their capacity for oligodendrocyte differentiation in the presence of inhibitory CSPGs

(Fig. 2.5J-K, Supplemental Fig. 2.5E-F). We observed a significant increase in the percentage of GFAP positive astrocytes and instead a marked decrease in Olig2 positive oligodendrocytes in our laminin+CSPGs experimental group which was entirely reversed by inhibition of the Rho/ROCK pathway. These results were complimented with western blotting where Y-27632 treatment restored GFAP and CNPase protein expression in NPCs grown on CSPGs to levels near that of laminin control group. Notably, Y-27632 treatment also promoted oligodendrocyte differentiation of NPCs on a laminin substrate compared to laminin only control condition, however, this increase was not statistically significant. Altogether, these results indicate that the Rho/ROCK pathway plays a central role in mediating the modulatory effects of CSPGs on several aspects of NPCs activities. *All values are provided in Supplementary Table 2.4*.

Figure 2.5. Activation of the Rho/ROCK pathway mediates CSPGs inhibitory effects on spinal cord NPCs.
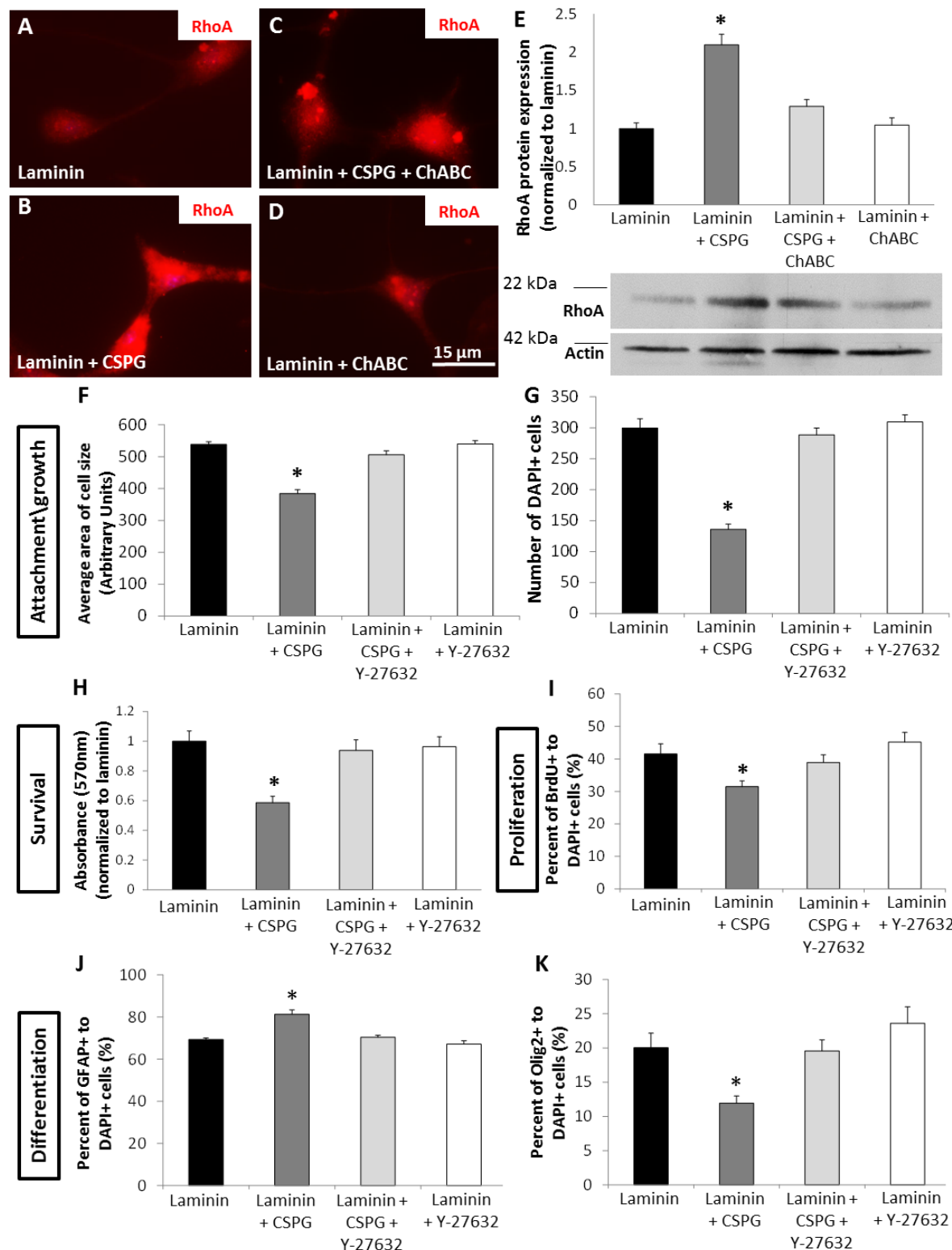
Figure 2.5. Activation of the Rho/ROCK pathway mediates CSPGs inhibitory effects on spinal cord NPCs.

NPCs were grown on laminin (10 µg/ml) or laminin (10 µg/ml) + CSPG (5 µg/ml) substrate and grown in growth media for 2 days or differentiated in 2% serum media for 7 days. (A-D) Immunostaining of NPCs revealed upregulation in the expression of RhoA in NPCs when grown on laminin+CSPGs substrate (B) in comparison to laminin alone. (E) A significant increase in the expression of RhoA protein was observed in NPCs grown on laminin+CSPGs compared to all other experimental conditions 7 days following cell plating. Involvement of the Rho/ROCK pathway in CSPGs negative effects on NPC growth, attachment and survival was demonstrated using Y-27632 (10µM), a ROCK inhibitor. (F-G) CSPGs inhibitory effects on cell spreading (F), attachment (G) and survival (H) was significantly overcome by Y-27632 treatment. (I) Y-27632 treatment overcame CSPGs inhibitory effect on NPC proliferation (J-K) Immunostaining of NPCs was completed measuring the percent of GFAP positive and Olig2 positive cells to the total number of DAPI positive cells. Y-27632 pretreatment of NPCs reversed CSPG effects on oligodendrocyte differentiation to a level closer to laminin condition with. (K) NPCs grown on laminin + Y-27632 alone slightly promoted the oligodendrocyte differentiation of NPCs however these results were not significant ($p = 0.204$ in comparison to laminin only). N = 4 independent experiments. The data show the mean ± SEM. * $P<0.05$, one-way ANOVA.

2.4.5 Erk and Akt phosphorylation plays a key role in CSPG effects on spinal cord NPCs

Recent studies show that interactions between RhoA and Akt pathways regulate the inhibitory effects of CSPGs on neurite outgrowth (Fisher et al. 2011). Here, we examined the same possibility in NPCs. Western blot analyses showed that exposure to CSPGs significantly decreased Akt phosphorylation in NPCs. There was a 49% reduction in the ratio of phosphorylated Akt (p-Akt) to total Akt (t-Akt) in NPCs plated on laminin+CSPGs substrate compared to laminin only substrate (Fig. 26A) which was reversed by ChABC pretreatment. We further demonstrated that CSPGs induce Akt dephosphorylation through both LAR and PTPσ receptors (Fig. 2.6B). We observed a 45% decrease in Akt phosphorylation in NPCs treated with control NC1 DsiRNA when plated on CSPGs whereas NPCs with downregulated LAR and RPTPσ only showed 8% and 17% decrease in their pAKT/tAKT ratio, respectively. Furthermore, we investigated whether the Rho/ROCK pathway mediated the CSPGs inhibitory effects on Akt phosphorylation (Fig. 2.6C). As shown in previous experiments, there was a significant 40% reduction in pAkt/tAkt ratio when NPCs were grown onto laminin+CSPGs, which was entirely overcome in NPCs pretreated with ROCK blocker Y-27632. Interestingly, ROCK inhibition also increased Akt phosphorylation in NPCs plated on laminin (134.92%) to levels higher than that of laminin alone (100%) and laminin+CSPGs+Y-27632 (118.16%) conditions indicating that suppressing Rho/ROCK pathway generally increases Akt activation in NPCs.

We further explored CSPG effects on the phosphorylated state of Erk1/2 (Fig. 2.6D-F). Reduced phosphorylation of Erk1/2 has been previously shown to correlate strongly with a decrease in oligodendrocytes differentiation and myelin thickness (Ashii et al. 2012; Fyffe-Maricich et al. 2011a; Guardiola-Diaz, Ishii, and Bansal 2012). Based on this evidence, we asked whether CSPGs inhibit oligodendrocyte differentiation in NPCs by suppressing Erk1/2

phosphorylation. We observed that the ratio of pErk1/2 to tErk1/2 was significantly decreased in NPCs grown on laminin+CSPGs substrate in comparison to laminin condition (Fig. 2.6D). Our DsiRNA studies showed that CSPGs cause Erk1/2 dephosphorylation primarily by signaling on RPTPσ (Fig 2.6E). We observed a 35% decrease in the ratio of pErk1/2 to tErk1/2 in control NC1 treated NPCs grown on laminin+CSPGs substrate. Downregulation of RPTPσ in NPCs attenuated Erk1/2 dephosphorylation to 13% compared to laminin substrate while LAR downregulation had no effects. These data indicate that signaling through RPTPσ but not LAR mediates the effects of CSPGs on the phosphorylation of Erk1/2 in NPCs. Lastly; we asked whether the Rho/ROCK pathways modulate the phosphorylation status of Erk1/2 in NPCs in response to CSPGs (Fig. 2.6F). To this end, our data demonstrated that Y-27632 treatment restored Erk1/2 phosphorylation in NPCs on CSPGs in laminin+CSPGs condition to levels comparable to NPCs plated on laminin or laminin+ Y-27632 suggesting that activation of Rho/ROCK may be linked to dephosphorylation of Erk1/2 in NPCs after exposure to CSPGs.

Figure 2.6. CSPGs limit the phosphorylation of Akt and Erk1/2 by signaling through LAR and RPTPσ and activation of the Rho/ROCK pathway.
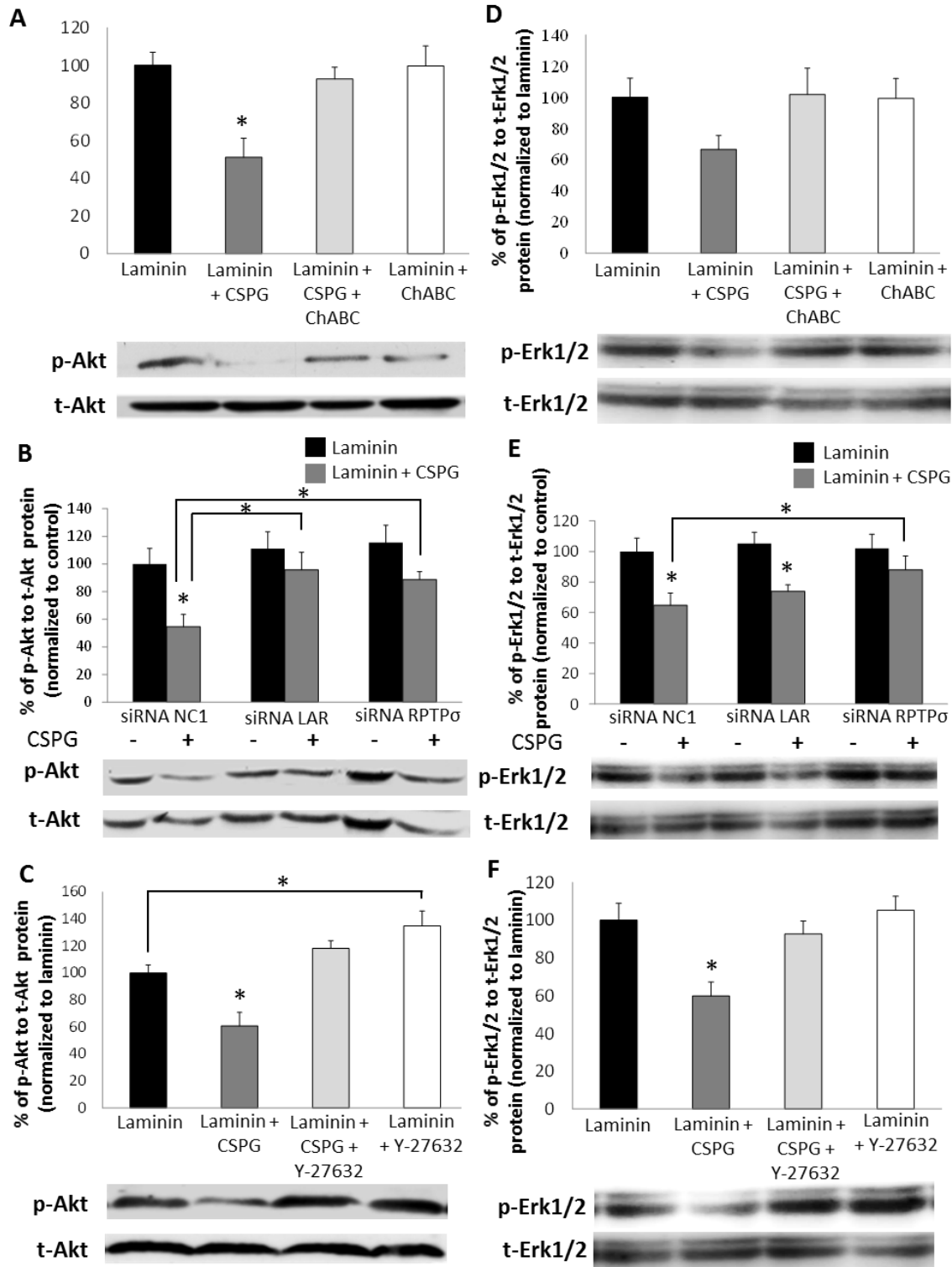
Figure 2.6. CSPGs limit the phosphorylation of Akt and Erk1/2 by signaling through LAR and RPTPσ and activation of the Rho/ROCK pathway.

NPCs were grown on laminin (10 μg/ml) or laminin (10 μg/ml) + CSPG (5 μg/ml) substrate and differentiated in 2% serum media for 7 days. (A) Western blotting analysis showed a significant decrease in the ratio of phosphorylated Akt (pAkt) to total Akt (tAKT) in NPCs exposed to laminin+CSPGs compared to laminin condition. (B) In control NC1 DsiRNA condition, NPCs had a significant decrease in the ratio of pAkt/tAkt when grown on laminin+CSPGs substrate which was significantly overcome through downregulation of LAR and RPTPσ. (C) Inhibition of the Rho/ROCK (Y-27632, 10μM) pathway attenuated CSPG effects on the phosphorylation state of Akt in NPCs grown on laminin+CSPGs substrate. Interestingly, the ratio of pAkt/tAkt was also significantly higher in laminin + Y-27632 condition. (D) Western blotting analysis showed decrease in the phosphorylation of Erk1/2 protein in NPCs grown on CSPGs compared to all control condition, although it did not reach a significant level ($p = 0.135$). (E) Downregulation of LAR and RPTPσ partially removed the inhibitory effects of CSPGs on the phosphorylated state of Erk1/2; however, this attenuation was only statistically significant in RPTPσ DsiRNA NPCs in comparison to control condition, DsiRNA NC1 NPCs when grown on laminin+CSPGs substrate. (F) Inhibition of the Rho/ROCK pathway attenuated CSPG effects on Erk1/2 phosphorylation in NPCs grown on laminin+CSPGs substrate. For all figures, N = 4 independent experiments. The data show the mean ± SEM. *(A,C,D,F) * P<0.05, one-way ANOVA, (B,E) *P<0.05, Student t test.*

2.5 Discussion

In the present study, we report that CSPGs directly and negatively regulate the behavior of spinal cord NPCs in a receptor-mediated manner. Using *in vitro* assays mimicking the ECM composition of injury, we demonstrate that exposure to CSPGs inhibits several cellular aspects of NPCs including their growth, integration, survival, proliferation, and oligodendrocyte differentiation. CSPGs exert these effects by signalling on both LAR and RPTPσ as well as activation of the Rho/ROCK pathway. At the intracellular level, activation of CSPGs signaling decreases the phosphorylated state of Akt and Erk1/2 in NPCs, which appear to be downstream mediators of CSPG effects on spinal cord NPCs. Our findings, for the first time, introduce the cellular mechanisms by which CSPGs may modulate the regenerative response of NPCs within the microenvironment of injury and identifies potential targeted interventions for efficient optimization of NPCs-based repair strategies for CNS injuries.

Application of NPCs holds a great promise for SCI repair (Karimi-Abdolrezaee and Eftekharpour 2012; Tetzlaff et al. 2011; Eftekharpour, Karimi-Abdolrezaee, and Fehlings 2008b; Karimi-Abdolrezaee et al. 2006b; Karimi-Abdolrezaee et al. 2010; Hofstetter, Holmström, et al. 2005; Parr et al. 2008; Ziv et al. 2006). However, recent studies indicate that the survival and multipotential capacity of CNS derived NPCs for cell replacement is restricted in the post-SCI milieu (Karimi-Abdolrezaee et al. 2006b; Karimi-Abdolrezaee et al. 2010; Hofstetter, Holmström, et al. 2005; Barnabé-Heider et al. 2010; Meletis et al. 2008). Our previous work in chronic SCI demonstrated a strong correlation between the injury-induced upregulation of CSPGs and the suboptimal proliferation and oligodendrocyte differentiation of transplanted and resident NPCs in spinal cord lesion (Karimi-Abdolrezaee et al. 2010) (Karimi-Abdolrezaee et al. 2012). Recently, CSPGs are also shown to inhibit the process outgrowth of oligodendrocyte precursor cells (OPCs)

and myelination *in vitro* (Pendleton et al. 2013; Lau et al. 2012b) and in demyelinating lesions (Lau et al. 2012b; Larsen et al. 2003b). Replacement of oligodendrocytes is an essential approach for remyelination and functional repair following SCI (Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006b). Here, we employed a direct *in vitro* system to model the ECM of SCI where laminin and CSPGs are highly elevated (Buss et al. 2009; Galtrey and Fawcett 2007). When exposed to a matrix containing CSPGs, adult NPCs showed a significant decrease in their ability to integrate, grow, proliferate and survive. Of note, recent evidence indicates that proper activation of the resident NPC population is required to limit the extent of lesion following SCI and release neurotrophic factors which improve neuronal survival and attenuate tissue degeneration (Sabelström et al. 2013).

We also found that CSPGs restricted the potential of NPCs to differentiate into oligodendrocytes and instead favoured generation of new astrocytes. Notably, our data suggest that the inhibitory effect of CSPGs on oligodendrocyte differentiation is an active process and not due to initial selective attachment of astrocytes to the substrate resulting in a decrease in oligodendrocyte differentiation. This possibility was eliminated by plating undifferentiated NPCs and allowing them to attach to the substrate prior to serum differentiation. Our findings also explain our *in vivo* observation where we showed a strong correlation between upregulation of CSPGs and increased differentiation of resident or transplanted NPCs towards astrocytic fate following SCI (Mothe and Tator 2005b; Barnabé-Heider et al. 2010; Meletis et al. 2008; Karimi-Abdolrezaee et al. 2012).

The recent discovery of CSPGs receptors, RPTPσ (Shen et al. 2009b; Fry, Chagnon, López-Vales, et al. 2010), LAR (Fisher et al. 2011), as well as Nogo receptor (NgR) family members, NgR1 and NgR3 (Dickendesher et al. 2012) allows new understandings of CSPGs

mechanism in CNS regeneration. LAR and RPTPσ are widely expressed in adult CNS neurons and are shown to mediate the inhibitory effects of CSPGs on axonal regeneration (Fisher et al. 2011; Shen et al. 2009b). Lack of RPTPσ promoted axon growth on CSPGs substrates *in vitro* (Shen et al. 2009b). RPTPσ knockout mice showed improved regeneration of the corticospinal tract following SCI (Shen et al. 2009b; Fry, Chagnon, López-Vales, et al. 2010) and enhanced axonal regeneration after optic nerve and peripheral nerve injuries (McLean et al. 2002; Thompson et al. 2003; Sapeiha et al. 2005). Blocking LAR also resulted in increased axonal growth of serotonergic fibers around and beyond the SCI lesion accompanied by functional recovery (Fisher et al. 2011). Here, our new evidence extends these concepts by revealing that spinal cord NPCs express both LAR and RPTPσ *in vitro* and *in vivo* and thereby their activities can be influenced directly by upregulation of CSPGs. Using siRNA gene silencing as well as RPTPσ knockout studies, we demonstrate that downregulation of LAR or RPTPσ partially reversed CSPGs inhibitory effects on NPCs with additive effects after their co-inhibition. Deletion of RPTPσ *per se* did not alter the potential of NPCs for self-renewal and differentiation which is in agreement with an earlier study (Kirkham et al. 2006). Interestingly, embryonic derived neuronal restricted precursor cells (NRPs) are intrinsically insensitive to CSPGs, and this was correlated with reduced expression of both RPTPσ and LAR in growth cones of NRP derived neurons (Ketschek et al. 2012).

We found that knockdown of LAR and/or RPTPσ did not entirely reverse CSPG effects on NPCs. This could be due to the transient siRNA-mediated knockdown of the LAR and RPTPσ, the compensatory action of LAR and RPTPσ and/or involvement of LAR and RPTPσ independent mechanisms of CSPGs in NPCs. CSPGs are additionally shown to inhibit axon growth by signaling on Nogo receptors, NgR1 and NgR3 (Dickendesher et al. 2012) and by blocking laminin/integrin

signaling (Zhou et al. 2006; Zuo et al. 1998). Importantly, aggrecan directly inhibits laminin-mediated axon growth by impairing integrin signaling through decreasing phosphorylated FAK and Src levels (Orlando et al. 2012; Tan et al. 2011). Notably, aggrecan was included in our CSPGs mixture and our substrate also contained laminin to more closely represent the ECM composition of SCI (Buss et al. 2009). Thereby, it is plausible that blockade of integrin/laminin signaling in NPCs partly underlined the inhibitory effects of CSPGs independent of LAR and/or RPTPσ. Our findings also indicate that knockdown of LAR and/or RPTPσ did not alter the behavior of spinal cord NPCs on laminin only substrate suggesting that these receptors are not involved in laminin signaling in NPCs. Collectively, our data demonstrates that the inhibitory role of LAR and RPTPσ in NPCs regulation is CSPGs specific.

At the intracellular level, we demonstrate a pronounced upregulation of RhoA protein expression in NPCs after exposure to CSPGs. Notably, we did not specifically look at RhoA activation levels in NPCs on CSPGs substrate. However, inhibition of the downstream ROCK was sufficient to reverse nearly all of CSPG effects on NPCs. Elevated levels of active RhoA are detected after axonal injury (Dubreuil, Winton, and McKerracher 2003; Conrad et al. 2005) resulting in growth cone collapse (Wahl et al. 2000; Monnier et al. 2003; Walker, Ji, and Jaffrey 2012; Maekawa et al. 1999). Moreover, Rho activation has been associated with p75 mediated apoptosis in neurons and glial cells following SCI (Dubreuil, Winton, and McKerracher 2003) which may be the underlying mechanism of the restored survival of NPCs on CSPGs following blockade of Rho/ROCK pathway in our experiments. CSPGs-LAR interaction also triggers the activation of RhoA in neurons (Fisher et al. 2011). Interestingly, CSPGs induced inhibition of oligodendrocyte myelination was also overcome by blocking ROCK and downregulation of RPTPσ (Pendleton et al. 2013; Siebert and Osterhout 2011b). Importantly, both CSPGs and myelin

associated inhibitors converge on RhoA pathway and inhibit axonal growth (Niederöst et al. 2002). Ba-210, a Rho inhibitor trademarked as Cethrin, is currently being assessed in clinical trials for SCI (Lord-Fontaine et al. 2008; Fehlings et al. 2011b) and our findings suggest that such strategy could be a potential combinatorial approach for promoting NPCs cell replacement activities following SCI.

Reduced phosphorylation state of both Akt and Erk1/2 in spinal cord NPCs through interaction with LAR and RPTPσ and the Rho/ROCK pathway (Fig. 2.7) may underlie the CSPG-mediated decrease in NPCs growth, adhesion, proliferation and differentiation in our studies. Activation of PI3K/Akt and/or MAPK/Erk pathways has been previously linked to induced NPCs proliferation (Chan et al. 2013) and enhanced oligodendrocytes survival and differentiation *in vitro* and *in vivo* (Flores et al. 2000; Rafalski et al. 2013; Rowe et al. 2012). Downstream effector of the PI3K/Akt, mTOR, is critical for oligodendrocyte differentiation (Tyler et al. 2009) and myelination (Flores et al. 2008; Goebbels et al. 2010). MAPK/Erk signaling also plays central role in oligodendrocyte process extension and myelination (Ashii et al. 2012; Furusho et al. 2012b; Fyffe-Maricich et al. 2011b; Flores et al. 2008) and our knockdown studies identified that CSPGs/RPTPσ signaling appears to be the key mediator of Erk1/2 dephosphorylation in NPCs (Fig. 2.6E). Altogether, our findings provide novel evidence that CSPGs signaling negatively regulates activation and oligodendrocyte differentiation of spinal cord NPCs likely by modulating their Akt and Erk1/2 activities.

Figure 2.7. Schematic illustration of proposed mechanisms of CSPGs in regulating spinal cord NPCs.

CSPGs modulate their inhibitory effects on adult spinal cord NPCs through multiple mechanisms. CSPG effects are directed through LAR and RPTPσ and mediated through the Rho/ROCK pathway and inhibition of the phosphorylation state of Akt and Erk1/2.

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2.6 Conclusion

In conclusion, the present study provides new direct insights into the mechanisms by which CSPGs restrict the regenerative activities of adult spinal cord NPCs. Upregulation of CSPGs is an inevitable outcome of CNS injuries with major impact on repair and regeneration. Here, we demonstrate that CPSGs modulate several properties of adult spinal cord NPCs through direct receptor-mediated mechanisms. Our findings suggest that blockage of LAR and RPTPσ signaling and/or the Rho/ROCK pathway in NPCs are plausible interventions for overcoming the inhibitory effects of CSPGs on cell replacement strategies. Knowledge gained in this study will aid in optimizing current cellular therapies for SCI and other CNS injuries characterized by the upregulation of CSPGs.

2.7 Acknowledgement

2.8 References

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2.9 Supplementary Materials

2.9.1 Supplementary Figures

Supplemental Figure 2.1. CSPGs inhibit the survival of adult spinal cord NPCs.
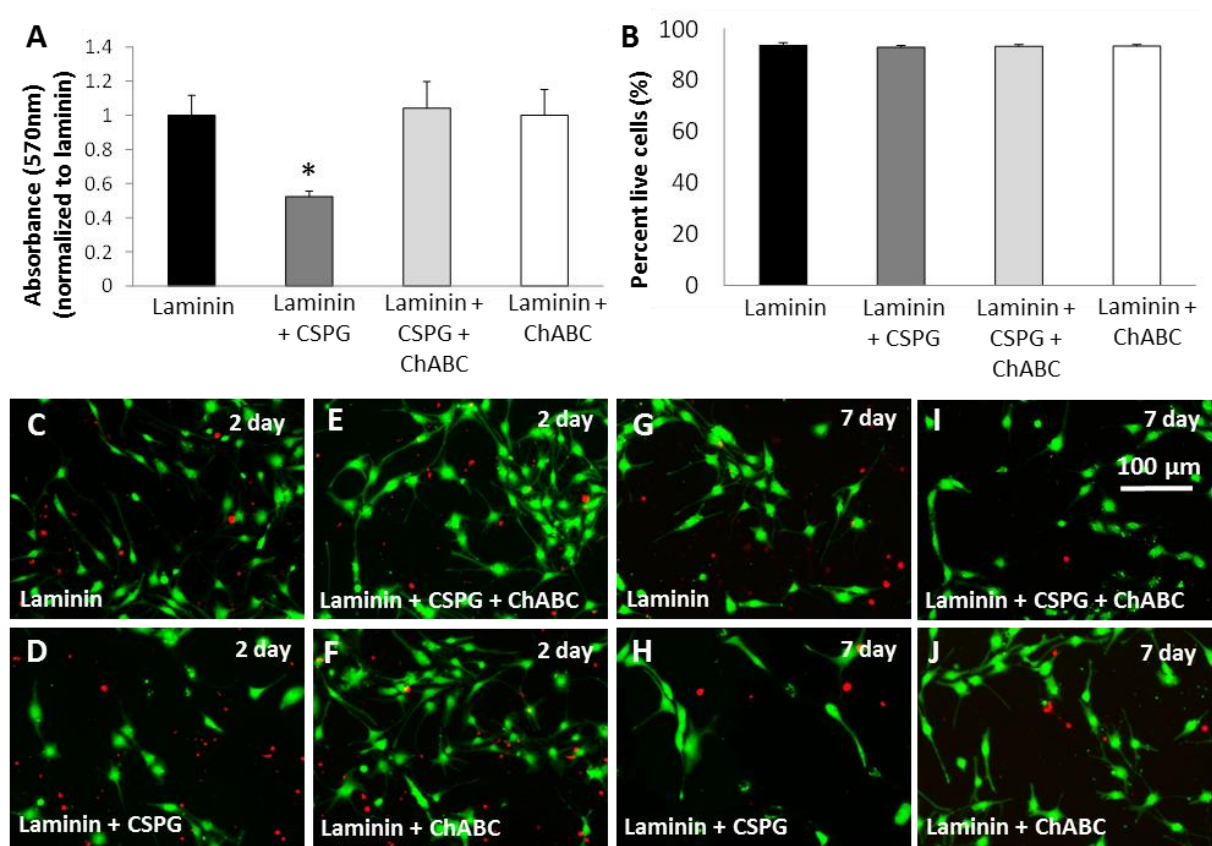
Supplemental Figure 2.1 CSPGs inhibit the survival of adult spinal cord NPCs.

Spinal cord NPCs were grown on laminin (10 µg/ml) or laminin (10 µg/ml) + CSPGs (5 µg/ml) substrate for 2 and 7 days following cell plating. (A) Survival of NPCs on CSPGs substrate was assessed using an MTT assay at 7 days showing a significant decrease in survival of NPCs grown on laminin+CSPGs substrate. (B-J) Assessment of NPC survival was complimented with a LIVE/DEAD assay using the same experimental parameters at 2 (C-F) and 7 days (G-J) following cell plating. Green cells were labelled as LIVE cells (Calcein) and red as DEAD cells (EthD-1). (B) There was no difference observed in the percentage of live cells at 7 days. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, one-way ANOVA.

Supplemental Figure 2.2. Downregulation of LAR and RPTPσ in spinal cord NPCs.

Supplemental Figure 2.2. Downregulation of LAR and RPTPσ in spinal cord NPCs.

 (A) Transfection of NPCs by DsiRNA was verified through TYE control showing incorporation of TYE into cell bodies. DsiRNA knockdown of LAR and RPTPσ was confirmed by qPCR and protein analysis. (B) There was a significant decrease in LAR mRNA expression in DsiRNA LAR and DsiRNA LAR + RPTPσ NPCs in comparison to DsiRNA NC1 control and DsiRNA RPTPσ NPCs. (C) Western blotting verified LAR knockdown showing a significant decrease in the expression of LAR protein in DsiRNA LAR and DsiRNA LAR + RPTPσ NPCs in comparison to other experimental conditions. (D) qPCR showed a significant decrease in RPTPσ mRNA expression in NPCs treated with DsiRNA RPTPσ and DsiRNA LAR + RPTPσ in comparison to NC1 control and DsiRNA LAR NPCs. (E) Western blotting confirmed RPTPσ knockdown showing a significant reduction in the expression of RPTPσ protein in DsiRNA RPTPσ and DsiRNA LAR + RPTPσ experimental groups. Note: In all figures, results from the experimental conditions were normalized to the control NC1 values, shown as 1 in the black bars. *N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, one-way ANOVA.*

Supplemental Figure 2.3. Cell Characterization of RPTPσ KO NPCs.

Supplemental Figure 2.3. Cell Characterization of RPTPσ KO NPCs.

NPCs from wild type and RPTPσ knockout NPCs were isolated from the subventricular zone of Balb/c mice and cultured in growth media for self-renewal assay or in serum media for differentiation assay. (A) Assessment of self-renewal of wild type and RPTPσ KO NPCs revealed no difference in the number of neurospheres generated 5 days following cell plating. (B) Additionally, no difference was observed in the neurosphere diameter of both wild type and RPTPσ-/- NPCs 5 days following cell plating. (C) Proliferation of NPCs was assessed examining the percentage of BrdU+/ DAPI+ cells at 2 days following cell plating in growth media. No change in the percentage of BrdU positive NPCs was found when comparing wild type and RPTPσ-/- NPCs from passages 2 to 5. (D-E) Differentiation assessment of wild type and RPTPσ-/- NPCs was observed 7 days following cell plating measuring the percentage of GFAP+/DAPI+ (D) and Olig2+/DAPI+ (E) cells. No apparent change in the differentiation pattern of NPCs was observed for both GFAP positive astrocytes (D) and Olig2 positive oligodendrocytes (E). NPCs harvested from RPTPσ-/- mice showed normal expression of LAR mRNA (F) or protein (G) comparable to the wild type NPCs. (H) Lack of RPTPσ mRNA expression in RPTPσ-/- NPCs was verified using qPCR. *Note: N = 8 independent experiments in figures A-B and F-H. N=4 independent experiments in figures C-E. The data show the mean ± SEM. *P<0.05, one-way ANOVA.*

Supplemental Figure 2.4. LAR and RPTPσ mediate the inhibitory effects of CSPGs on NPCs.

Supplemental Figure 2.4. LAR and RPTPσ mediate the inhibitory effects of CSPGs on NPCs.

(A-H) Bright field images of NPCs treated with different DsiRNA and grown on laminin (10 µg/ml) or laminin (10 µg/ml) + CSPGs (5 µg/ml) substrate 2 days following cell plating. NPCs treated with non-target control condition, DsiRNA NC1, showed limited attachment when grown on laminin+CSPGs (B) compared to those plated on laminin (A). Downregulation of LAR (C-D) and RPTPσ (E-F) partly attenuated CSPG effects on NPC attachment. Greater attenuation of CSPGs inhibitory effects was achieved when both LAR and RPTPσ were downregulated simultaneously. (I-J) LAR and RPTPσ mediate CSPGs inhibitory effect on oligodendrocyte differentiation (I) Western blotting demonstrated reduced GFAP protein expression in differentiated NPCs on laminin+CSPGs substrate after downregulation of LAR and RPTPσ in comparison to NC1 control. (J) In contrast, there was a significant increase in CNPase protein expression in DsiRNA LAR NPCs and DsiRNA RPTPσ grown on laminin+CSPGs substrate in comparison to DsiRNA NC1 NPCs plated on laminin+CSPGs substrate. (K) Survival of NPCs on CSPGs substrate was assessed using a LIVE/DEAD assay 2 days following cell plating. Downregulation of LAR or RPTPσ significantly improved the survival of NPCs when grown on CSPGs substrate in comparison to NC1 control group. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, Student *t* test.

Supplemental Figure 2.5. Activation of the Rho/ROCK pathway mediated CSPGs inhibitory effects on the growth, attachment, survival and oligodendrocyte differentiation of spinal cord NPCs.

Supplemental Figure 2.5. Activation of the Rho/ROCK pathway mediated CSPGs inhibitory effects on the growth, attachment, survival and oligodendrocyte differentiation of spinal cord NPCs.

(A-D) Bright field images of spinal cord NPCs grown on laminin (10 µg/ml) or laminin (10 µg/ml) + CSPG (5 µg/ml) substrate 2 days following cell plating. NPCs plated on laminin+CSPGs (B) showed limited cell spreading compared to laminin (A). (C) Treatment of NPCs with Y-27632 (10µM) attenuated CSPG effects on NPC attachment. (E-F) Y-27632 promoted the oligodendrocyte differentiation of NPCs when grown on laminin+CSPGs substrate. Western blotting showed a significant increase in the expression of GFAP protein in NPCs grown on laminin+CSPGs substrate a significant decrease in CNPase protein expression compared to all other experimental groups. Inhibition of Rho/ROCK signaling attenuated the effects of CSPGs and restored the level of GFAP and CNPase protein back to that of our control condition. (G) Survival of NPCs on CSPG substrate was assessed using a LIVE/DEAD assay 2 days following cell plating. Y-27632 treatment promoted the survival of NPCs when grown on CSPG substrate. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, one-way ANOVA.

2.9.2 Supplementary Tables

Supplementary Table 2.1: Values obtained in experiments studying CSPG effects on NPCs.

	Figure	Laminin	Laminin + CSPG	Laminin + CSPG + ChABC	Laminin + ChABC
Cell Growth/Spreading (N = 4)	Fig 2.1E	539.48 ± 11.93 (1 ± 0.02)	345.74 ± 12.46 (0.64 ± 0.02)	521.38 ± 17.48 (0.97 ± 0.03)	550.93 ± 4.82 (1.02 ± 0.01)
Cell Attachment (N = 4)	Fig 2.1F	296.50 ± 20.48 (1 ± 0.07)	157.25 ± 26.16 (0.53 ± 0.09)	308.75 ± 13.25 (1.04 ± 0.04)	299.50 ± 26.37 (1.01 ± 0.09)
Cell Survival- MTT 2 day (N = 4)	Fig 2.1G	1 ± 0.07	0.62 ± 0.03	1.01 ± 0.06	1.01 ± 0.07
Cell Survival- LIVE/DEAD Assay, 2 days (N=4)	Fig 2.1H	86.31% ± 1.43	68.71% ± 2.78	85.04% ± 1.79	85.04 ± 1.61
Cell Survival- MTT 7 days (N=4)	Supp. Fig 2.1A	1 ± 012	0.52 ± 0.03	1.04 ± 0.16	1.00 ± 0.15
Cell Survival- LIVE/DEAD Assay, 7 days (N=4)	Supp. Fig 2.1B	93.447% ± 1.12	92.75% ± 0.59	93.13% ± 0.72	93.19% ± 0.63
Cell Proliferation- BrdU (N=4)	Fig 2.1I	43.55% ± 2.45	32.73% ± 2.23	43.36% ± 2.64	42.71% ± 1.88
Cell Proliferation- Ki67 (N=4)	Fig 2.1N	40.73% ± 2.44	29.19% ± 3.78	39.69% ± 1.53	40.10% ± 1.77
Cell Differentiation- GFAP (N = 4)	Fig 2.2A	68.92% ± 1.30	82.04% ± 1.50	72.29% ± 1.19	70.14% ± 1.31
Differentiation- GFAP protein expression (N=4)	Fig 2.2F	1 ± 0.04	1.23 ± 0.10	0.99 ± 0.04	0.99 ± 0.07
Cell Differentiation- Olig2 (N = 4)	Fig 2.2G	19.86% ± 1.55	10.60% ± 1.38	17.50% ± 1.09	18.89 ± 1.19
Differentiation- CNPase protein expression (N=4)	Fig 2.2L	1 ± 0.06	0.53 ± 0.02	0.96 ± 0.06	0.98 ± 0.04

Supplementary Table 2.2: Experimental values obtained in DsiRNA analyse

	Fig.	siRNA NC1 NPCs		siRNA LAR NPCs		siRNA RPTPσ NPCs		siRNA LAR + RPTPσ NPCs	
		Laminin	Laminin + CSPG	Laminin	Laminin + CSPG	Laminin	Laminin + CSPG	Laminin	Laminin + CSPG
Cell Growth/Spreading (N = 4)	Fig 2.3K	622.39 ± 10.53 (1 ± 0.02)	393.17 ± 15.50 (0.63 ± 0.02)	630.55 ± 26.64 (1.01 ± 0.04)	504.68 ± 32.18 (0.81 ± 0.05)	628.20 ± 32.32 (1.01 ± 0.05)	537.76 ± 35.99 (0.86 ± 0.06)	626.40 ± 11.75 (1.01 ± 0.02)	576.49 ± 7.40 (0.93 ± 0.01)
Cell Attachment (N = 4)	Fig 2.3L	289.75 ± 27.02 (1 ± 0.09)	138.00 ± 10.93 (0.48 ± 0.04)	288.75 ± 15.93 (0.99 ± 0.05)	221.00 ± 17.18 (0.76 ± 0.06)	285.00 ± 13.20 (0.98 ± 0.05)	242.00 ± 11.01 (0.84 ± 0.04)	303.75 ± 31.21 (1.05 ± 0.11)	270.00 ± 21.98 (0.93 ± 0.08)
Cell Survival-MTT 2 days (N = 4)	Fig 2.3M	1 ± 0.05	0.59 ± 0.03	1 ± 0.04	0.75 ± 0.04	1 ± 0.05	0.82 ± 0.05	1 ± 0.05	0.90 ± 0.05
Cell Survival-LIVE/DEAD Assay, 2 days (N=4)	Supp. Fig 2.3K	89.15% ± 1.69	67.68% ± 1.48	87.86% ± 0.87	76.77% ± 2.39	88.93% ± 0.94	74.76% ± 0.77	-	-
Cell Survival-MTT 7 days (N=4)	-	1 ± 0.03	0.58 ± 0.03	1 ± 0.06	0.75 ± 0.03	1 ± 0.14	0.82 ± 0.09	1 ± 0.09	0.90 ± 0.05
Cell Survival-LIVE/DEAD Assay, 7 days (N=4)	-	92.65% ± 2.69	92.40% ± 2.24	92.66% ± 1.92	92.82% ± 2.26	92.83% ± 2.26	92.68% ± 1.54	-	-
Cell Proliferation-BrdU (N=4)	Fig 2.3E	38.45% ± 1.68	26.35% ± 1.63	38.44% ± 3.41	31.80 ± 2.83	38.35% ± 3.94	33.48% ± 2.91	38.66% ± 1.46	35.90% ± 1.11
Cell Proliferation-Ki67 (N=4)	-	36.43% ± 1.48	26.31% ± 1.21	36.19% ± 2.56	31.06% ± 2.31	35.98% ± 2.43	31.78% ± 3.02	36.80% ± 1.57	34.60% ± 1.31
Cell Differentiation-GFAP (N = 4)	Fig 2.3O	69.90% ± 1.77	79.53% ± 1.83	70.16% ± 1.70	73.47% ± 1.39	69.50% ± 1.30	72.46% ± 1.56	-	-
Differentiation-GFAP protein expression (N=4)	Supp. Fig 2.4I	1 ± 0.04	1.22 ± 0.04	0.95 ± 0.03	1.10 ± 0.07	1.01 ± 0.04	1.12 ± 0.05	-	-
Cell Differentiation-Olig2 (N = 4)	Fig 2.3P	22.52% ± 1.63	12.74% ± 0.48	22.048% ± 1.13	18.14% ± 0.85	22.80% ± 1.12	18.97% ± 0.89	-	-
Differentiation-CNPase protein expression (N=4)	Supp. Fig 2.4J	1 ± 0.06	0.61 ± 0.08	1.07 ± 0.10	0.88% ± 0.07	1.11 ± 0.09	0.90 ± 0.07	-	-

Supplementary Table 2.3: Values obtained in experiments on RPTPσ -/- mouse.

	Figure	Wild Type NPCs		RPTPσ Knockout NPCs	
		Laminin	Laminin + CSPG	Laminin	Laminin + CSPG
Cell Growth/Spreading (N = 4)	Fig 2.4A	287.75 ± 27.15 (1 ± 0.03)	153.75 ± 17.44 (0.69 ± 0.03)	298.75 ± 19.15 (1.01 ± 0.02)	236.00 ± 19.49 (0.85 ± 0.02)
Cell Attachment (N = 4)	Fig 2.4B	559.59 ± 14.13 (1 ± 0.04)	385.45 ± 19.54 (0.53 ± 0.03)	566.35 ± 13.08 (1.04 ± 0.05)	477.06 ± 10.54 (0.82 ± 0.04)
Cell Survival- MTT 2 days (N = 4)	Fig 2.4C	1 ± 0.09	0.61 ± 0.05	1 ± 0.05	0.86 ± 0.04
Cell Proliferation- BrdU (N = 4)	Fig 2.4D	42.12% ± 2.14	27.68% ± 1.22	41.19% ± 2.23	36.71% ± 2.71
Cell Differentiation- GFAP (N = 4)	Fig 2.4E	1	1.17 ± 0.05	1.00 ± 0.03	1.06 ± 0.01
Cell Differentiation- Olig2 (N = 4)	Fig 2.4F	1	0.54 ± 0.04	1 ± 0.04	0.89 ± 0.01

Supplementary Table 2.4: Values obtained in Rho/ROCK experiments.

	Figure	Laminin	Laminin + CSPG	Laminin + CSPG + Y-27632	Laminin + Y-27632
Cell Growth/Spreading (N = 4)	Fig 2.5F	538.64 ± 8.29 (1 ± 0.02)	384.29 ± 12.59 (0.71 ± 0.02)	505.88 ± 12.38 (0.94 ± 0.02)	539.99 ± 10.33 (1.00 ± 0.02)
Cell Attachment (N = 4)	Fig 2.5G	299.50 ± 15.12 (1 ± 0.05)	136.00 ± 8.60 (0.45 ± 0.03)	288.50 ± 11.03 (0.96 ± 0.04)	309.50 ± 11.44 (1.03 ± 0.04)
Cell Survival- MTT 2 days (N = 4)	Fig 2.5H	1 ± 0.07	0.59 ± 0.04	0.94 ± 0.07	0.96 ± 0.07
Cell Survival- LIVE/DEAD Assay, 2 days (N=4)	Supp. Fig 2.5G	89.51% ± 2.10	67.60% ± 2.53	80.14% ± 2.77	89.48% ± 1.89
Cell Survival- MTT 7 days (N=4)	-	1 ± 0.11	0.50 ± 0.06	0.89 ± 0.09	0.97 ± 0.10
Cell Survival- LIVE/DEAD Assay, 7 days (N=4)	-	94.27% ± 0.93	94.80% ± 0.48	94.83% ± 0.76	94.50% ± 0.28
Cell Proliferation- BrdU (N=4)	Fig 2.5I	41.56% ± 3.06	31.46% ± 1.82	38.93% ± 2.31	45.17% ± 3.00
Cell Proliferation- Ki67 (N=4)	-	37.16% ± 1.16	28.28% ± 1.16	35.52% ± 1.20	40.82% ± 1.20
Cell Differentiation- GFAP (N = 4)	Fig 2.5J	69.33% ± 0.75	81.29% ± 2.05	70.39% ± 0.94	67.12% ± 1.60
Differentiation- GFAP protein expression (N=4)	Supp. Fig 2.5E	1 ± 0.08	1.12 ± 0.11	1.03 ± 0.06	0.91 ± 0.07
Cell Differentiation- Olig2 (N = 4)	Fig 2.5K	20.04% ± 2.13	11.94% ± 1.03	19.58% ± 1.60	23.60% ± 2.42
Differentiation- CNPase protein expression (N=4)	Supp. Fig 2/5F	1 ± 0.05	0.57 ± 0.09	0.99 ± 0.07	1.22 ± 0.07

Chapter 3: Inhibition of LAR and PTPσ Signaling Promotes Endogenous Oligodendrocyte Replacement and Preservation following Spinal Cord Injury

This chapter has been prepared as a manuscript that will be submitted to Glia in March 2018 after addition of myelination data that are currently in final preparation.

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Authors contributions: I contributed to over 80% of the total experimental procedures, data and analysis, and manuscript preparation. Cell culture of Neural Stem/Precursor Cells was done by Santhosh Thomas Kallivalappil and oligodendrocyte precursor cell cultures were done by Hardeep Kataria.

Conflict of Interest: S.D, H.K, A.A, and S.K-A declare no competing financial interests. J.S is the inventor on the patent application (PCT/US2013/035831) for ISP.

3.1 Abstract

Repair of spinal cord injury (SCI) is challenged by substantial cell death and an impermissible microenvironment for cell replacement. The population of mature oligodendrocytes undergoes substantial cell death following injury; promoting their preservation and replacement is a viable strategy for SCI. Dramatic upregulation of matrix chondroitin sulfate proteoglycans (CSPGs) is known to pose an obstacle to multiple endogenous repair processes including oligodendrocyte replacement. Targeting CSPGs improves functional recovery after SCI; however, the cellular and molecular mechanisms underlying their inhibitory effects in SCI remain largely undefined. The discovery of CSPGs specific receptors, leukocyte common antigen-related (LAR) and protein tyrosine phosphatase-sigma (PTPσ), allows us to uncover CSPGs direct mechanisms in regulating oligodendrocytes in SCI. Here, in a clinically relevant model of rat contusive/compressive SCI, we demonstrate that pharmacological inhibition of PTPσ and LAR signaling with specific functionally blocking peptides attenuates caspase 3 mediated cell death in mature oligodendrocytes and preserves myelin after injury, while promoting oligodendrogenesis by endogenous precursor cells. In parallel *in vitro* systems, we have unraveled that CSPGs directly induce apoptosis in populations of neural precursor cells and oligodendrocyte progenitor cells, and limit their ability for oligodendrocyte differentiation. These negative effects of CSPGs are mediated through both LAR and PTPσ receptors, and activation of the downstream Rho/ROCK pathway. Thus, we have identified a novel inhibitory role for CSPGs receptors, PTPσ and LAR, in regulating oligodendrocyte differentiation and apoptosis in the injured adult spinal cord, and a new feasible therapeutic strategy for optimizing endogenous cell replacement following SCI.

3.2 Introduction

The extent of spontaneous recovery is limited following spinal cord injury (SCI) due to a multitude of factors that include an imbalanced inflammatory response, degeneration of neurons, axons and oligodendrocytes, and the formation of an inhibitory glial scar (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015; Fawcett 2002). Replacing damaged oligodendrocytes either through cell transplantation or activation of endogenous precursor populations is a viable therapeutic target for SCI repair (Alizadeh and Karimi-Abdolrezaee 2016). Despite a spontaneous oligogenic response by endogenous neural precursor cells (NPCs) and oligodendrocyte precursor cells (OPCs) after SCI, the number of newly formed oligodendrocytes is insufficient to overcome the extent of oligodendrocyte loss and demyelination. (Karimi-Abdolrezaee et al. 2006; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Mothe and Tator 2005; Barnabe-Heider et al. 2010; Ikegami et al. 2005; Almad, Sahinkaya, and McTigue 2011; Horky et al. 2006). Currently, the regulatory mechanisms of oligodendrocyte replacement in the post-SCI milieu are not fully understood.

Following SCI, the extracellular matrix (ECM) is altered profoundly where reactive astrocytes encapsulate the lesion site and deposit chondroitin sulfate proteoglycans (CSPGs) within the glial scar (Cregg et al. 2014; Dyck and Karimi-Abdolrezaee 2015). CSPGs severely impede axonal plasticity and regeneration (Lang et al. 2015; Alilain et al. 2011; Galtrey et al. 2007; Bradbury et al. 2002; Dyck and Karimi-Abdolrezaee 2015; Barritt et al. 2006). We have also identified a negative role for CSPGs in restricting the survival, integration and oligodendrocyte differentiation of adult NPCs in cell transplantation approaches for SCI (Karimi-Abdolrezaee et al. 2010). Importantly, ample evidence from preclinical studies by our group and others demonstrates that inhibition of CSPGs as a solitary approach or in combinatorial therapies can

improve functional recovery in SCI (Bradbury et al. 2002; Fouad et al. 2005; Karimi-Abdolrezaee et al. 2010; Lang et al. 2015). However, our understanding of the cellular and molecular mechanisms underlying CSPGs inhibitory role in limiting the endogenous cell response following SCI is limited.

The discovery of two specific CSPGs signaling receptors, leukocyte common antigen-related (LAR) and protein tyrosine phosphatase-sigma (PTPσ) (Shen et al. 2009; Fisher et al. 2011), provides the opportunity for targeted manipulation of CSPGs associated pathways. Our previous in vitro studies showed that spinal cord NPCs highly express LAR and PTPσ receptors and, thereby, their regenerative properties can be modulated by the presence of CSPGs in their injury niche (Dyck et al. 2015). Genetic manipulation of LAR and PTPσ receptors in cultures of spinal cord NPCs identified a novel inhibitory role for these receptors in mediating the detrimental effects of CSPGs on NPC differentiation (Dyck et al. 2015). Currently, the role and mechanisms of LAR and PTPσ signaling in regulating endogenous oligodendrogenesis and oligodendrocyte integrity in the injured adult spinal cord is largely unknown.

Recent work by our group and others demonstrated that LAR and PTPσ can be efficiently manipulated in a clinically-relevant model of rat contusive SCI by membrane permeable blocking peptides named Intracellular LAR Peptide (ILP) and Intracellular Sigma Peptide (ISP), respectively, resulting in improved functional recovery (Fisher et al. 2011; Lang et al. 2015). Interestingly, we found that targeting PTPσ promotes axon regeneration and notably remarkable serotonergic re-innervation below the level of SCI that contributes to functional recovery (Lang et al. 2015). However, pharmacological blockade of serotonergic receptors was not sufficient to entirely reverse functional improvement of ISP treatment suggesting additional contributing endogenous mechanisms. Here, utilizing ILP/ISP in SCI-relevant *in vitro* and *in vivo* models, we

have identified a novel critical role for LAR and PTPσ receptors in the injured spinal cord, which negatively impacts oligodendrocyte integrity, differentiation, and maturation during the repair processes following injury. Our findings also provide new understanding to the cellular and molecular mechanisms by which modulation of LAR and PTPσ receptors can improve neurological recovery following SCI (Lang et al. 2015; Fisher et al. 2011).

3.3 Materials and Methods

3.3.1 Animals and Animal care

All experimental protocols in this study were approved by the Animals Care Committee of the University of Manitoba in accordance with the guidelines and policies established by the Canadian Council of Animal Care (CCAC). For *in vivo* studies, a total of 112 adult female Sprague Dawley (SD) rats (250g), and for *in vitro* experiments, 20 C75BL/6 mice (8 weeks of age) were used for NPCs cultures and 16 rat pups (1-3 days of age) were used for OPC cultures (provided by the Central Animal Care Facility at the University of Manitoba, Canada). For SCI, adult female rats were housed in standard plastic cages at 22°C before their injury and at 26°C afterwards in a 12:12 h light/dark cycle. Pelleted food and drinking water were available *ad libitum*. Hardwood sawdust bedding was used before SCI surgeries and replaced by soft paper bedding after SCI to prevent skin erosions and urine scalding. For post-surgical pain and discomfort management, immediately after surgery, each animal received a combination of buprenorphine (Vetergesic, 0.03mg/kg) and meloxicam (Metacam® Boehringer Ingelheim GmbH, 2mg/kg) followed by three additional doses of buprenorphine with 8 hour (hr) intervals. SCI rats were examined daily to monitor their recovery and their bladder was expressed manually three times a day until the return of reflexive bladder control.

3.3.2 Isolation and Culturing of Spinal Cord Adult NPCs

Adult NPCs were isolated from the spinal cord of C57BL/6 mouse (8 weeks of age) as we described previously (Dyck et al. 2015; Gauthier et al. 2013). Briefly, mice were deeply anesthetized by placing in a bell jar saturated with a mixture of 40% isoflurane/60% propylene glycol. Deep anesthesia was confirmed by lack of pedal reflexes. Mice were then killed by decapitation, and the spinal cord was excised under sterile conditions and transferred to artificial cerebrospinal fluid (aCSF) solution (containing 124mM NaCl, 3mM KCl, 1mM NaHPO4, 26mM NaHCO3, 1.5mM MgSO4, 1.5mM CaCl2, and 10 mM glucose). The spinal cord was cleaned of meninges and nerve roots and subject to papain enzymatic solution (Worthington Biochemical Corporation) for 50 min at $37^{\circ}C$. The solution was next replaced by a papain inhibitor mixture and cells were passed through a 70µm cell strainer. Cellular components were isolated through 7.5% BSA gradient and resuspended in serum free medium (SFM, 100 ml) containing 84ml of DMEM/F12 (Invitrogen), 2ml of 30% glucose, 1.5ml of 7.5% $NaHCO_3$, 0.5ml of 1M HEPES, 10mg of transferrin, 2.5mg of insulin, 0.96mg of putrescine, 1µl of selenium, 1µl of progesterone, 1% L-glutamine, 1% penicillin/streptomycin/neomycin (PSN) and growth factors: 1µg of fibroblast growth factor-2 (FGF2, Fisher, CB40060A), 2µg of epidermal growth factor (EGF, Sigma, E-4127), and 200µg of Heparin (Sigma, H-3149). SFM plus growth factors will be referred as growth medium in the text. Cells were plated onto uncoated tissue culture flasks (Biolite, Fisher Scientific). The neurospheres generated were passaged weekly by mechanical dissociation in growth medium.

3.3.3 Plating Spinal Cord NPCs onto Laminin and CSPGs Substrates

This procedure was conducted as we previously described (Dyck et al. 2015). Briefly, tissue culture dishes were first coated with poly-D-lysine (PDL, 0.1mg/ml, Sigma) overnight at room temperature, followed by laminin (5µg/mL, Sigma, L2020) and/or CSPGs (5µg/mL, Millipore, cc117) for 6 hrs at 37°C as we described previously (Dyck et al. 2015). Of note, CSPGs used in this study contained a mixture of neurocan, phosphacan, versican, and aggrecan. For NPC plating, neurospheres were dissociated into single cells and plated onto coated tissue culture surfaces (12,000 cells per cm^2). The NPC assessments, the cells were grown in either growth medium for cell attachment and proliferation, or in serum medium (SFM plus 2% fetal bovine serum (FBS), Invitrogen) to induce differentiation (7 day assessments). For proliferation assessment, BrdU (20µM, Sigma) was added to the cultures 3 hrs before processing NPCs for immunocytochemistry.

3.3.4 Primary culture of cortical OPCs

Primary OPCs cultures were isolated from the cerebral cortex of P1-2 rats as we described previously (Kataria et al. 2018). Briefly, cerebral cortices were dissected out and enzymatically dissociated in a solution containing papain (0.9 mg/ml; Worthington Biochemical), L-cysteine (0.2 mg/ml; Sigma), EDTA (0.2 mg/ml; Sigma) diluted in HBSS (Invitrogen) for 45 minutes, at 37°C. Digested tissue was filtered through a 40µm cell nylon strainer (BD Biosciences) and seeded on poly-D-lysine (PDL) coated flasks in DMEM medium supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin-neomycin (PSN, Invitrogen). The cultures were maintained at 37°C and 5% CO_2 and culture medium was changed every 3 days. When the cultures reached confluence, approximately after 10 days, they were shaken overnight at 250 rpm at 37°C. Medium was then filtered through 40µm cell nylon strainer (BD Biosciences) and centrifuged at

1000 rpm for 10 minutes. Cells were then seeded in uncoated Petri dishes (BD Biosciences) to eliminate microglial cells. After 30min, media was collected and centrifuged for 10 mins at 1000rpm to isolate OPCs. These cells were then plated onto various conditions as described below.

3.3.5 Plating cortical OPCs onto Laminin and CSPGs substrate

Primary cortical OPCs were plated onto PDL (0.1mg/ml), laminin 5µg/mL) and/or CSPGs (10µg/mL) coated multi-chamber glass slides (15,000 cells per chamber) (LabTek) in OPC culture medium. Coating procedure was performed as described above for NPCs. Where appropriate, chondroitinase ABC (ChABC, 0.1 U/ml Sigma, C3667-10UN) was added with laminin + CSPGs mixture during the coating step to degrade CSPGs. After 24h of plating, culture media was changed to OPC differentiation medium [DMEM (Sigma), insulin (10 µg/mL), transferrin (50 µg/mL), sodium selenite (5.2 ng/mL), hydrocortisone (18 ng/mL), putrescine (16 µg/mL), progesterone (6.3 ng/mL), biotin (10 ng/mL), N-acetyl-L-cysteine (5 µg/mL), BSA (0.1%), and PSN (Invitrogen), 1% FBS, 5 µM of forskolin] for 10 days. For attachment, survival and proliferation assessments, OPCs were seeded in OPC differentiation medium (without forskolin and FBS) for 24h. For proliferation assessment, BrdU (20µM, Sigma) was added to the cultures 3 hrs before processing OPCs for immunocytochemistry.

3.3.6 *In vitro* Assessment of ILP and ISP Peptides in Blocking CSPG effects on NPCs and OPCs

ILP and ISP peptides, against LAR and PTPσ, respectively, were used as we described previously (Lang et al. 2015). ILP and ISP binds to a highly conserved 24-amino acid intracellular wedge domain of LAR and PTPσ and block the catalytic activity of the receptor (Lang et al. 2015; Xie et

al. 2006). Efficacy of ILP and ISP in blocking CSPG effects has been verified and reported in vitro and in SCI (Lang et al. 2015; Xie et al. 2006; Fisher et al. 2011). Intracellular Mu Peptide (IMP, NH2-LLQHITQMKCAEGYGFKEEYESGRKKRRQRRRC-NH2, CS Bio Co.) (Xie et al. 2006), a control peptide, was also used to assess specificity of ILP and ISP effects. All peptides contained a transactivator of transcription of human immunodeficiency (TAT), domain (GRKKRRQRRRC) to facilitate intracellular delivery. To determine the efficacy and optimal dosing of ISP and ILP in NPCs and OPCs, we performed *in vitro* dosing assays by examining NPCs/OPCs attachment and cell spreading on CSPGs substrate (assessment described below). Spinal cord derived NPCs or rat pup derived OPCs were plated onto tissue culture surfaces containing one of the two conditions: 1) Laminin; 2) Laminin + CSPGs. For both experimental conditions, NPCs or OPCs were pretreated with IMP (control), TAT peptide (control), ILP, ISP, or ILP and ISP. We used various concentrations of the peptides including 1.25μM, 2.5μM, 5μM, 10μM and/or 15μM. The 10μM concentration was determined to be the optimal dose for both ILP and ISP in NPCs (Supplemental Fig. 3.1), and the 2.5μM concentration was determined to be the optimal dose for OPCs (Supplemental Fig. 3.2). These concentrations were used for all *in vitro* experimental conditions thereafter. Additionally, our previous work in NPCs demonstrated CSPG effects are mediated through activation of the Rho/ROCK pathway (Dyck et al. 2015). As a result, in our OPC cultures, we treated them with Y-27632 (10μM), a ROCK inhibitor, to investigate the intracellular mechanisms of CSPG effects on OPCs.

3.3.7 Assessment of Cell Attachment and Spreading in Primary NPC and OPCs Cultures

One day after NPC or OPCs plating on laminin and/or CSPGs, cells were fixed with 3% paraformaldehyde (PFA). The slides were washed three times with PBS and incubated with the

nuclear marker 4, 6-diamidino-2-phenylindole (DAPI, 1:5,000 in PBS) for 15 minutes. Slides were coverslipped with Mowiol mounting medium. Assessment of cell attachment and spreading was performed as we previously described (Dyck et al. 2015). Briefly, Using StereoInvestigator Cavalieri probe, (MBF Bioscience), we performed unbiased measurement of the total area of cells and their processes in 8-10 separate bright field and fluorescent images (under 20X objective) containing an average of 300 cells for each treatment condition. To estimate the average growth area occupied by each NPC/OPC, the total area was divided by the total number of DAPI positive cells in each field as we reported previously (Dyck et al. 2015).

3.3.8 Assessment of NPC and OPC Survival through MTT and LIVE/DEAD Assays

These assessments were performed according to our previously described methods (Dyck et al. 2015).

3.3.8.1 *MTT Assay:* Dissociated NPCs were plated in 96-well plates at density of 30,000 cells/well in growth medium for a 2-day assessment. For MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay, 10 µl of MTT reagent was added to each well and incubated at 37°C for 3 hours. Without removing the media, cells were lysed and formazan crystals formed in the mitochondria were solubilized by adding 100ul of 100% DMSO to each well. Absorbance was measured at a wavelength of 570nm with a reference filter at 690nm by a spectrophotometer.

3.3.8.2 *LIVE/DEAD Assay:* NPCs or OPCs were plated on laminin and/or CSPG coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) for 1 day. Cell viability was assessed by a LIVE/DEAD assay according to manufacturer instructions (Life Technologies). Images of 8-10 randomly chosen fields were taken under 20x objective with a Zeiss fluorescent microscope with 494nm (green, Calcein) and 528nm (red, EthD-1) excitation filters. For analysis, the

percentage of live cells was determined by quantifying the total number of LIVE (green) cells compared to the total number of LIVE (green) and DEAD (red) cells in analyzed fields.

3.3.9 *In vitro* Immunostaining, Image Processing and Analysis

For immunocytochemistry, cultures were fixed with 3% paraformaldehyde (PFA) for 20 minutes at room temperature and washed three times with PBS. Cells were incubated in a blocking solution containing 5% non-fat milk, 1% BSA, and 0.5% Triton X-100 in 0.1M PBS for 1 hr. Cultures then underwent immunostaining procedure similar to methods described earlier for immunohistochemistry. For BrdU immunodetection, prior to blocking, sections were washed with PBS, incubated in 2N HCl and 0.5% Triton X-100 for 30 minutes at 37°C, and washed with 0.1M sodium borate in PBS for 10 minutes. After blocking, the slides were then incubated with primary antibodies overnight and secondary antibodies were added as was previously described. For immunocytochemistry quantification, 8-10 separate fields (under 20X objective) containing an average of 300 cells for each condition were randomly imaged using a Zeiss AxioObserverZ1 inverted microscope or a Zeiss Imager 2 epi-fluorescence microscope. For each condition, the total number of DAPI positive cells was first assessed, and the number of positive cells for Olig2, GFAP, Cleaved Caspase 3, MBP, O4, and BrdU (containing a DAPI positive nucleus) were then counted. The percentage of abundance for each cell type was calculated by dividing the number of positive cells for the marker by the total number of DAPI+ cells under each experimental condition. Values were then normalized to control condition for relative comparison.

3.3.10 Rat Model of Compressive Spinal Cord Injury

We employed a clinically relevant clip-compression model of SCI that has been extensively characterized and employed for SCI pathophysiology and therapeutics by our group and others (Rivlin and Tator 1977; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Gauthier et al. 2013). Under sterile conditions, general anesthesia was induced by inhalation of a mixture of O_2 (2 liters per min) and Isoflurane (4%) via a mask integrated into a surgical stereotaxic frame. After deep anesthesia was achieved, for maintenance, isoflurane was reduced to 2%. The surgical area was shaved and disinfected with 70% ethanol and Povidone Iodine. A midline incision was made at the thoracic area (T4–T9) and skin and superficial muscles were retracted. The rats received a T6–T8 laminectomy and then, a 35g aneurysm clip (University Health Network, Toronto, Ontario, Canada) was applied extradurally for 1 min at the level of T7 of the spinal cord inducing a compression injury. SCI animals were given postoperative analgesia including buprenorphine (Vetergesic, 0.03mg/kg) and Meloxicam (2mg/kg) as well as 5 ml of 0.9% saline subcutaneously to prevent dehydration. Additionally, animals received oral Clavamox® (Amoxicillin plus Clavulanic Acid, Pfizer) in their drinking water starting two days before surgeries until 3 days post-operation to prevent trauma-induced hematuria and bladder infection.

3.3.11 Experimental Groups and Treatments

In rat SCI, we first performed a dosing assay by immunohistochemical analyses and evaluated 3 daily doses of ILP (NH2-GRKKRRQRRRCDLADNIERLKANDGLKFSQEYESI-NH2, CS Bio Co.) and ISP (NH2-GRKKRRQRRRCDMAEHMERLKANDSLKLSQEYESI-NH2, CS Bio Co.) including 5μg, 10μg and 15μg per day. For dosing studies, rats received treatment at the time

of SCI for either 7 days (N = 4 animals/group) or 28 days (N = 5-6 animals/group) intrathecally in a sustained fashion using a mini-osmotic pump and an indwelling intrathecal catheter inserted into the subarachnoid space surrounding the lesion site with the tip of the catheter located in the rostral region to the lesion (Table 3.1). Prior to the SCI procedure, animals were randomly assigned to four experimental groups: 1) SCI/vehicle control, receiving vehicle solution used for preparation of ILP + ISP (0.1% bovine serum albumin, BSA in 0.9% saline); 2) SCI/ ILP + ISP (5µg each/day); 3) SCI/ ILP + ISP (10µg each/day); 4) SCI/ ILP + ISP (15µg each/day) (Table 3.1). Once optimal dosing was determined to be 10µg/day of ILP and ISP, we conducted extended time-point analyses at 1, 3, 5, 7 and 14 days in three experimental groups: 1) Uninjured; 2) SCI/vehicle control; 3) SCI/ ILP+ISP (10µg each/day). For all time-points (Table 3.1), treatment was administered intrathecally at the time of SCI using a mini-osmotic pump (Alzet –model 2001D, 1003D, 2001, 2002 and 2004). Based on the delivery rate of each pump, ILP and ISP concentration was adjusted to ensure sustained delivery of comparable dose per day for each time-point. SCI/vehicle control group also received vehicle via osmotic pumps in the same manner as the treatment groups.

Table 3.1. Summary of experimental procedures, treatment groups, time-points, dosing and sample size for *in vivo* experiments.

Histological Analyses

SCI ILP + ISP or vehicle treatment starts at the time of injury

Time-points for histological analysis

7 days

Duration of treatment delivery after SCI

28 days

Day 0

BrdU administration daily for 14 days

Experimental Groups:

1) SCI + Vehicle (saline + BSA)

2) SCI + ILP (5µg/day) + ISP (5µg/day)

3) SCI + ILP (10µg/day) + ISP (10µg/day)

4) SCI + ILP (15µg/day) + ISP (15µg/day)

Sample size:

N= 4 rats/group for 7 day study; N = 5-6 rats/group for 28 day study

Molecular Analyses

SCI ILP + ISP or vehicle treatment starts at the time of injury

Acute/Subacute Study

Time-points for molecular analysis of tissue lysate

Day 0   1 day   3 days   5 days   7 days   14 days

Duration of treatment delivery after SCI

Western Blot: 1, 3, 5, 7 and 14-day post injury

Experimental Groups:

1) Uninjured Control

2) SCI + Vehicle (saline + BSA)

3) SCI + ILP (10µg/day) + ISP (10µg/day)

Sample size:

N= 4-6 rats/group for acute time-points study

3.3.12 BrdU Incorporation Regimen

To examine the proliferation kinetics and fate of the mitotically active cells in the injured spinal cord after treatments, all rats received daily subcutaneous injection of 5-Bromo-2-deoxyuridine (BrdU, IP, 50mg/kg) starting immediately following injury for up to 14 days post-injury as we described previously (Karimi-Abdolrezaee et al. 2012; Gauthier et al. 2013).

3.3.13 Tissue Processing

Tissue harvesting was performed at the end of treatment period. Animal that received treatment at the time-point of injury were euthanized at 1, 3, 5, 7, 14 and 28 days post-SCI (N = 4-6 animals/group/time-point). SCI rats were deeply anesthetized with a mixture of 40% isoflurane/60% propylene glycol (Fisher Scientific, Pittsburgh, PA, USA). For molecular analyses, rats were perfused transcardially with 0.1M phosphate buffered saline (PBS) to remove blood. Once blood was removed, freshly dissected spinal cords were placed in ice-cold aCSF, and cleaned of meninges and nerve roots. Five millimeters of the spinal cord tissue centered at the injury site was dissected and processed either for Western blotting or slot blotting, as described in subsequent sections. For histological analyses, under deep anesthesia, rats were perfused transcardially with 2.5% paraformaldehyde (PFA) in 0.1M PBS, pH 7.4. The spinal cords were excised and subsequently postfixed in the perfusing solution plus 10% sucrose overnight at 4°C. Then, the tissues were further cryoprotected in 20% sucrose in PBS for 24-48 hrs at 4°C. A 1.5-2 cm length of the spinal cord tissue centered at the injury site was dissected and embedded in tissue embedding medium (Tissue-Tek™ CRYO-O.C.T Compound, Electron Microscopy Sciences) on dry ice. Cross sections (35 µm) were cut serially on a cryostat (Leica) and mounted onto Superfrost® Plus Micro Slides (Fisher Scientific) and stored at -80°C until immunostaining procedure.

3.3.14 Immunohistochemistry on Tissue Sections

Frozen spinal cord cross-sections were air-dried at room temperature for 30 minutes. The injury epicenter for each sample was determined by Hematoxylin and Eosin (H/E) staining and the section near the midpoint of the lesion with the largest injury area was considered as the epicenter. Slides were permeabilized with PBS for 5 minutes and then blocked for 1 hr at room temperature using 5% skim milk, 1% BSA, 0.05% Triton-X in 0.1M PBS. This blocking solution was used for all immunostaining procedures unless otherwise has been mentioned. Tissue sections were then incubated overnight at 4°C with primary antibodies (Table 3.2) diluted in the blocking solution. Sections were washed three times in PBS then incubated with Alexa 568 goat anti-mouse, rabbit, or goat secondary antibody (1:400; Invitrogen) for 1.5 hr. In double staining procedure, the tissue sections were treated with a second primary antibody then incubated with Alexa 488 goat anti-mouse or rabbit secondary antibody (1:400; Invitrogen). The slides were washed three times with PBS and incubated with DAPI as a nuclear counterstain. For BrdU immunodetection, sections were washed with PBS then, incubated in 2N HCl and 0.5% Triton X-100 for 30 minutes at 37°C, and washed with 0.1M sodium borate in PBS for 10 minutes. After blocking, the slides were then incubated with mouse anti-BrdU overnight and secondary antibodies were added as we previously described (Gauthier et al. 2013). Of note, BrdU immunostaining and acid treatment was always conducted after all previous primary and secondary antibodies were incubated on the tissue.

Table 3.2. List of antibodies used in this study

Note: ICC: immunocytochemistry, IHC: immunohistochemistry, WB: Western blot

Antibody	Source	Usage	Dilution factor
Actin	Sigma (Rabbit, A2066)	WB	1:300
Actin	Chemicon (Mouse, MAB1501R)	WB	1:300
pan Akt	Cell Signaling (Rabbit, 4691)	WB	1:500
p-Akt	Cell Signaling (Rabbit, 2965)	WB	1:500
APC	EMD Millipore (Mouse, OP80)	IHC	1:50
BrdU	Santa Cruz (Mouse, 555627)	ICC IHC	1:500 1:400
BrdU (5% BSA blocking)	Abcam (Rat, ab6326)	IHC	1:400
Cleaved Caspase 3	Cell Signaling (Rabbit, 9664)	WB ICC IHC	1:1000 1:300 1:300
CS56	Sigma (Mouse, C8035)	WB	1:500
DAPI	Sigma (D9542)	ICC IHC	1:10000
Erk1/2	Cell Signaling (Rabbit, #4695S)	WB	1:1000
p-Erk1/2	Cell Signaling (Rabbit, #4370S)	WB	1:1000
GFAP	Cell Signaling (Mouse, #3670S)	WB	1:5000
GAPDH	Santa Cruz (Rabbit, sc-25778)	WB	1:1000
MBP	Covance (Mouse, SMI-94R)	IHC	1:1000
MBP	Millipore (Rabbit, AB980)	ICC	1:200
O4	R&D (Mouse , MAB1326)	ICC	1:200
Olig2	Chemicon (Rabbit, AB9610)	ICC IHC	1:2000 1:1000

3.3.15 Assessment of MBP Immunodensity

MBP immunostaining was imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) (N = 4-6 animals/group). Four images of the white matter (lateral funiculi, dorsal funiculus and anterior funiculus) using a 20x objective lens. Immunodensity measurement was performed using NIH ImageJ software (imagej.nih.gov) as we described previously (Alizadeh 2017; Karimi-Abdolrezaee et al. 2010).

3.3.16 Assessment and Analysis of Endogenous Cell Response and Oligodendrocyte Cell Death

We used confocal microscopy to acquire images of the multi-fluorescent labelled sections of the spinal cord. For quantification of cell proliferation and differentiation, we examined two different cross sections of the spinal cord between 1–3 mm rostral to the injury center where all injured rats had an intact central canal and ependymal layer (N = 4–7 rats/group). Using images taken by confocal microscopy (Zeiss 710LSM) at 25x primary magnification, we first counted the number of BrdU-positive cells in 6 specified regions per spinal cord cross section (368 mm x 368 mm) that included the ependymal/subependymal region, dorsal and lateral columns and ventral gray and white matter. Then, we calculated the percentage of co-labeled BrdU+/Olig2+, BrdU+/APC+, or BrdU+/GFAP+ among all BrdU + cells in each spinal cord section. The same imaging techniques and analyses were performed for assessment of oligodendrocyte cell death by looking at the total number of APC+/Cleaved Caspase-3+ cells. In all neuroanatomical procedures, quantifications were executed in an unbiased manner by examiners blinded to the treatment groups based on the previously described methods by our group (Gauthier et al. 2013; Karimi-Abdolrezaee et al. 2010).

3.3.17 Western and Slot Blotting

For Western blotting, spinal cord tissue or cultured cells were homogenized in RIPA buffer (Thermo Fisher) containing SigmaFast Protease Inhibitor (Sigma). A total of 10µg to 50µg of proteins were then loaded on gel and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were then blocked in 5% non-fat milk in Tween Tris Buffered Saline (TTBS) and incubated overnight at 4ºC with different antibodies (Table 3.2) diluted in the blocking solution. The membranes were washed and incubated with HRP-conjugated goat anti-mouse, anti-sheep, anti-rat or anti-rabbit antibodies (1:4000, Bio-Rad). Membranes were then incubated in ECL plus immunoblotting detection reagents (Thermo Scientific) according to the manufacturer specifications. To control for equal protein loading, membranes were re-probed for actin antibody. Slot blotting was conducted for detection of CSPGs with 5µg of protein of SCI tissue per sample on a nitrocellulose membrane using Bio-Dot® slot blot system (Bio-Rad). The membrane was then blocked and incubated with mouse-anti CS56s for 2 hours at room temperature. The membranes were developed in the same manner as described for Western blotting.

3.3.18 Statistical Analysis

Using SigmaStat Software (4.0), we performed two-way repeated measures analysis of variance (ANOVA) followed by Holm-Sidak post-hoc test for distance analysis in immunohistological assessment in SCI. One-way ANOVA followed by Holm-Sidak post-hoc was used in all slot blot, immunocytochemistry and Western blot analyses. Student t-test was used when two groups were compared. The data was reported as means ± standard error of the mean (SEM). $P \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 ILP and ISP Efficiently Block CSPGs Inhibitory Effects on NPCs in an Additive Manner

We previously reported that CSPGs impede several properties of spinal cord NPCs including their attachment, growth, survival, proliferation, and oligodendrocyte differentiation by signaling through both LAR and PTPσ (Dyck et al. 2015). NPCs highly express LAR and PTPσ and genetic knockdown of both receptors was sufficient to reverse the inhibitory effects of CSPGs on NPCs properties to a level closer to the control condition (Dyck et al. 2015). Here, we tested the efficacy of ILP and ISP in blocking LAR and PTPσ mediated effects of CSPGs on NPCs. We first determined the optimal dosage of ILP and ISP in blocking CSPG effects on NPC using our cell attachment and spreading assay (Supplemental Fig. 3.1). Dissociated primary adult spinal cord NPCs were grown onto substrates containing either laminin or a combination of laminin and CSPGs (laminin + CSPG) for one day (N= 3). Of note, laminin and CSPGs are highly upregulated in the ECM of the injured spinal cord and therefore their co-presence closely recapitulates the matrix composition of SCI (Dyck and Karimi-Abdolrezaee 2015; Didangelos et al. 2016). Additionally, we used a relevant CSPG substrate containing a mixture of neurocan, phosphacan, versican, and aggrecan that are all present in the ECM of the injured spinal cord. Our stereology-based measurement of the total occupied area of NPCs cell bodies and processes showed a significant 36.5% decrease in the ability of NPCs to grow their processes on CSPGs substrate, which was significantly overcome by both ILP and ISP treatment in a concentration dependent manner (Supplemental Fig. 3.1A-L; Fig. 3.1A-H, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Similarly, we observed a 64.9% decrease in the number of attached DAPI+ NPCs on a laminin + CSPGs substrate compared to laminin alone, which was also reversed by both ILP and

ISP treatments (Supplemental Fig. 3.1M; Fig 3.1B, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Our dosing analyses verified the efficacy of ILP and ISP in blocking the effects of CSPGs on NPCs at a concentration of 10μM in culture. Accordingly, for the remaining NPC *in vitro* studies, we used the optimized dosage of 10μM for both ILP and ISP. As a control for the specificity of ILP and ISP peptides, we also treated NPCs with IMP (10μM), an intracellular peptide against PTPμ, another member of the PTP receptor family (Lang et al. 2015). In addition, since ISP and ILP peptides are TAT conjugated, we examined the effects of TAT peptide (10μM) itself on NPCs. These control conditions verified that the specificity of ILP/ISP as control IMP or TAT peptides were not able to block CSPG effects on NPCs attachment and cell spreading (Fig. 3.1A-H). Following these proof-of-concept experiments, we performed a series of *in vitro* studies to further examine the effects of ILP/ISP treatment on the survival, proliferation and differentiation of NPCs after exposure to CSPGs.

Figure 3.1: ILP and ISP promote attachment and cell spreading of NPCs on CSPGs substrate.
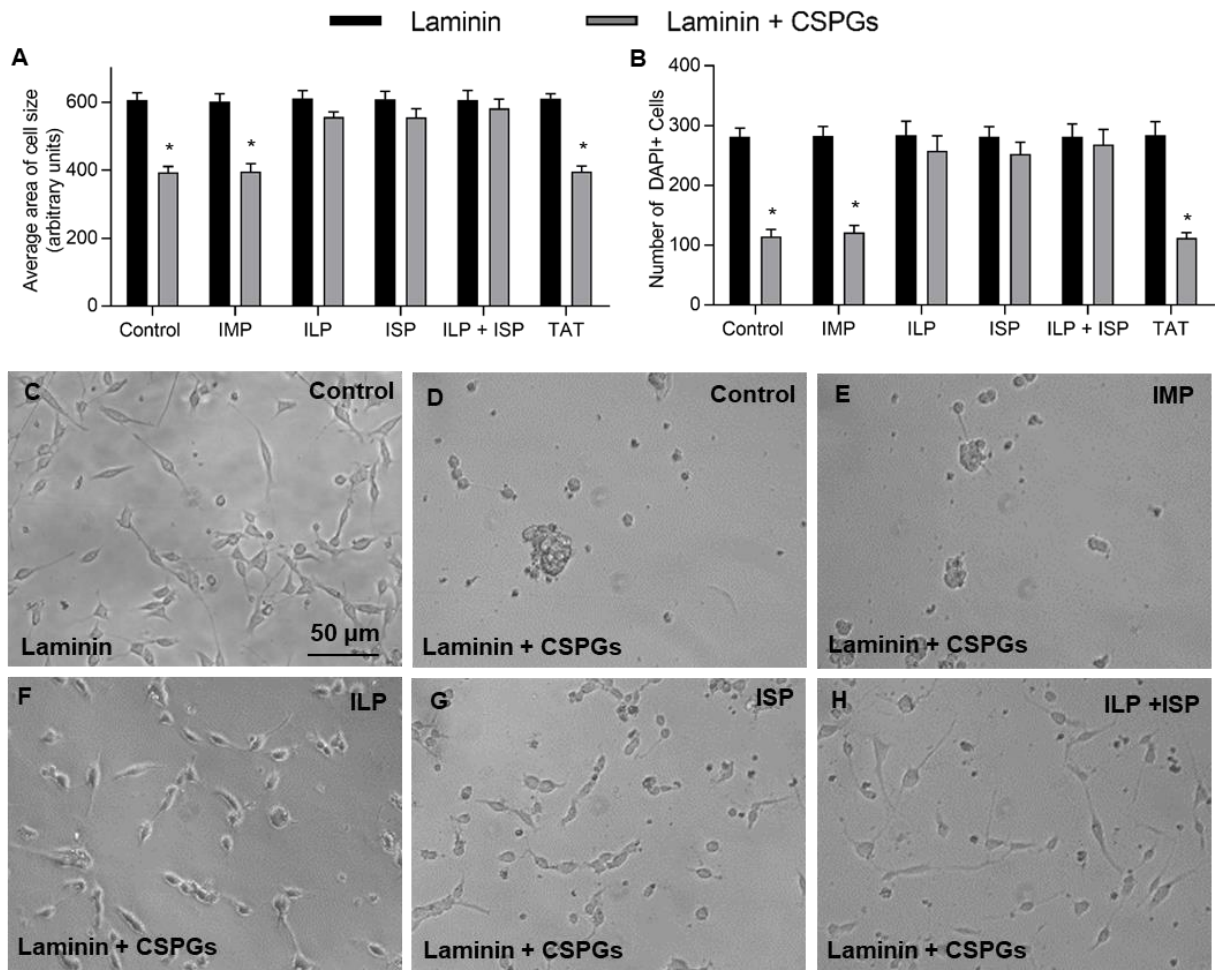
Figure 3.1: ILP and ISP promote attachment and cell spreading of NPCs on CSPGs substrate.

(A-H) NPCs cell spreading and attachment was assessed by measuring the total cell surface area (A) and number of DAPI+ cells (B). NPCs on laminin + CSPGs substrate (D) showed limited spreading compared to laminin only (C) control condition. ILP (F) and ISP (G) treatment improved the ability of NPCs to spread their processes and attach on inhibitory CSPGs substrate. (H) Co-treatment of ISP and ILP resulted in slight but non-significant improvement in cell spreading and NPC attachment on CSPGs substrate. Control peptides IMP and TAT showed no beneficial role in blocking CSPG effects on NPCs cell spreading or attachment indicating the specificity of ILP and ISP's effects on NPCs. N = 3-4 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

3.4.1.1 ILP/ISP mitigate CSPGs induced cell death in NPCs: We previously showed that CSPGs limit the survival of NPCs both *in vitro* (Dyck et al. 2015) and *in vivo* following transplantation into the injured spinal cord (Karimi-Abdolrezaee et al. 2010). Here, we show that inhibition of LAR and PTPσ with ILP and/or ISP promote NPC survival in the presence of CSPGs using complementary MTT (Fig. 3.2A) and LIVE/DEAD assays (Fig. 3.2B-N). The MTT assay showed that CSPGs significantly decreased NPC survival by 51% which was overcome by ILP or ISP treatments but not by TAT (20μM) or IMP control peptides. Co-treatment with ILP/ISP further improved NPC viability on CSPGs to a level closer to laminin control conditions with only a 10% decrease in their survival. Similar to our MTT data, our complementary LIVE/DEAD assay of NPCs survival showed a significant decrease in the percentage of live cells when NPCs were grown on CSPGs substrate. Solitary treatment of ILP and ISP significantly increased the percentage of live NPCs which was further improved by their combinatorial treatment. In addition, we found that exposure to CSPGs induces caspase 3 mediated apoptosis in NPCs, which was significantly overcome by ILP or ISP (Fig. 3.2O-R, N = 4, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Importantly, combinatorial use of ILP and ISP further attenuated CSPG effects on apoptosis, which was significantly greater than ILP or ISP treatment alone. Interestingly, we found that CSPGs induce apoptosis in NPCs by activation of Rho/ROCK signaling as the ROCK inhibitor Y-27632 was sufficient to attenuate cleaved caspase 3 expression in NPCs.

Figure 3.2: CSPGs limit NPCs survival and proliferation which is overcome by inhibition of LAR and PTPσ receptors and the Rho/ROCK pathway.
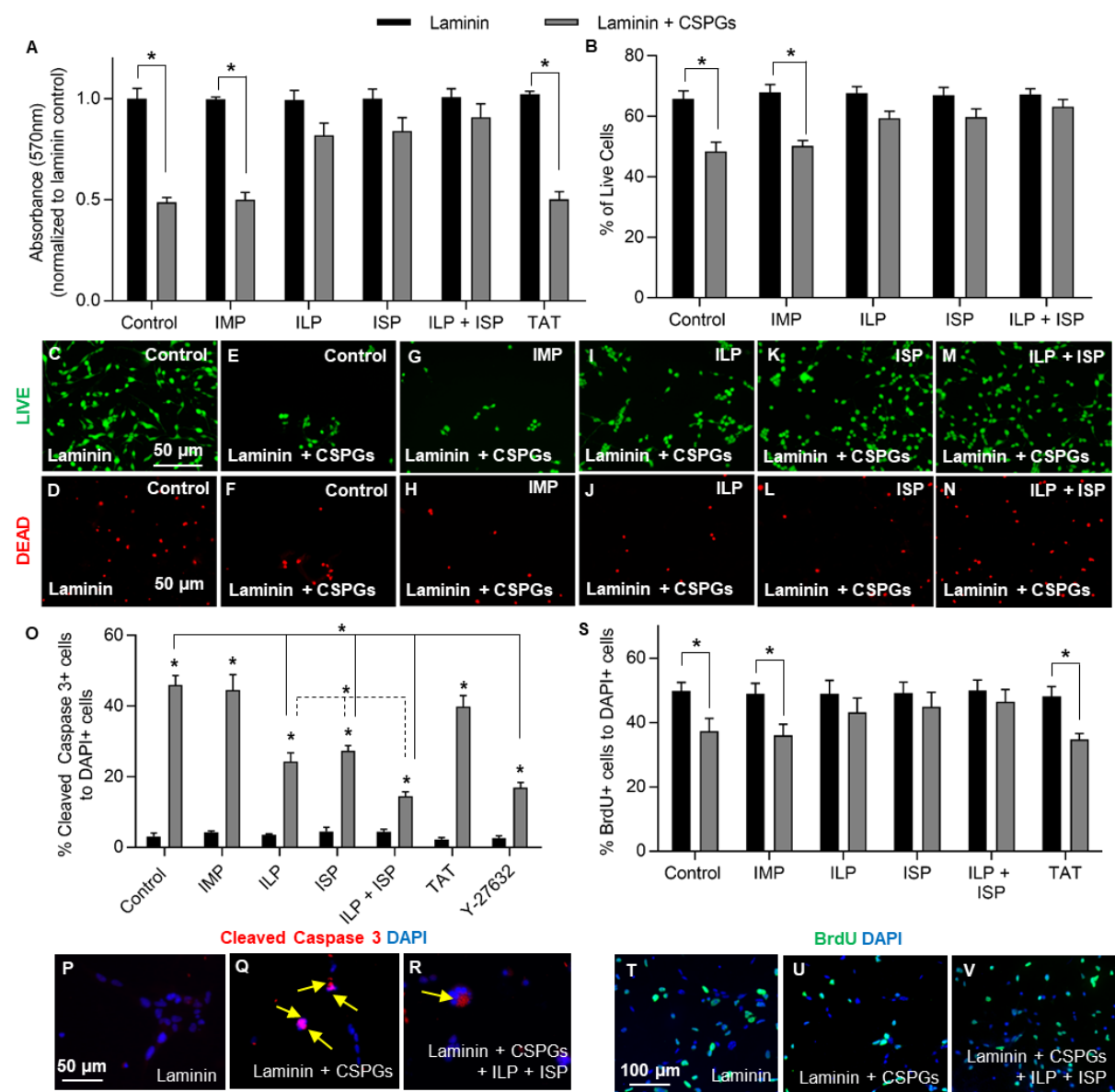
Figure 3.2: CSPGs limit NPCs survival and proliferation which is overcome by inhibition of LAR and PTPσ receptors and the Rho/ROCK pathway.

(A) Survival of NPCs on CSPGs substrate was assessed using an MTT assay at 2 days following cell plating showing a significant decrease in survival of NPCs grown on CSPGs substrate in non-treated TAT and IMP control groups. ILP and ISP treatment significantly improved NPC survival when exposed to CSPGs. Co-treatment with ILP and ISP further promoted the survival of NPCs on CSPGs substrate. (B-N) Assessment of NPC survival was complimented with a LIVE/DEAD assay using the same experimental parameters at 1 day following cell plating. Green cells were labelled as LIVE cells (Calcein) and red as DEAD cells (EthD-1). There was a significant decrease in the percentage of live NPCs when grown on laminin + CSPGs substrate for 1 day in both control and IMP experimental groups, however, ILP (I-J), ISP (K-L), and a combination of ILP and ISP (M-N) promoted the survival of NPCs on CSPG substrate. (O) In addition, CSPGs (Q) significantly increase the percentage of cleaved caspase 3 positive cells compared to laminin control (P) which was significantly overcome by ILP, ISP, or the ROCK inhibitor Y-27632 treatment. Co-treatment with ILP and ISP (R) further attenuated CSPG effects on caspase 3 mediated cell death, which was significantly greater than both ILP and ISP alone. (S-V) Using quantitative immunocytochemistry, we assessed NPCs proliferation. We calculated the percentage of BrdU+ proliferating cells to the total number of DAPI+ cells. Exposure to CSPGs (S) caused a significant decrease in proliferating NPCs (BrdU+/DAPI+) compared to NPCs grown on laminin alone (T). ILP, ISP and their combination (V) sufficiently blocked CSPG effects on NPC proliferation. N = 3-4 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

3.4.1.2 ILP/ISP attenuates the inhibitory effects of CSPGs on NPCs proliferation and differentiation: Ability of multipotent NPCs to proliferate and differentiate is a prerequisite for successful replacement of damaged spinal cord cells following injury (Karimi-Abdolrezaee and Eftekharpour 2012; Barnabe-Heider and Frisen 2008). Using BrdU incorporation that labels proliferating cells, we found a 26% decrease in proliferation of spinal cord derived NPCs when exposed to CSPGs. This effect was overcome by both ILP and ISP but not by control peptides IMP or TAT. Co-treatment of ILP/ISP more pronouncedly promoted NPCs proliferation on CSPGs substrates. ILP/ISP had an effect on the baseline proliferation of NPCs on laminin control condition (Fig. 3.2S-V, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*).

Following SCI, oligodendrocytes are subject to degeneration and their replacement is vital for axon remyelination. Previously, we demonstrated that CSPGs drives the fate of NPCs towards an astrocytic fate by signaling through both LAR and PTPσ while limiting their potential for oligodendrocyte differentiation (Dyck et al. 2015). Here, ILP and ISP treatment allowed NPCs to overcome the CSPGs inhibition on oligodendrocyte differentiation (Fig. 3.3A-N). When exposed to CSPGs, NPCs showed a significant 17% increase in their astrocyte differentiation (GFAP+/DAPI+) accompanied by a 37% decrease in oligodendrogenesis (Olig2+/DAPI+). Treatment with ILP or ISP improved oligodendrocyte differentiation by NPCs, but once again, ILP/ISP co-treatment more effectively blocked CSPG effects on NPCs differentiation ($p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc)*.

Figure 3.3: ILP and ISP promote oligodendrocyte differentiation on inhibitory CSPGs substrate.
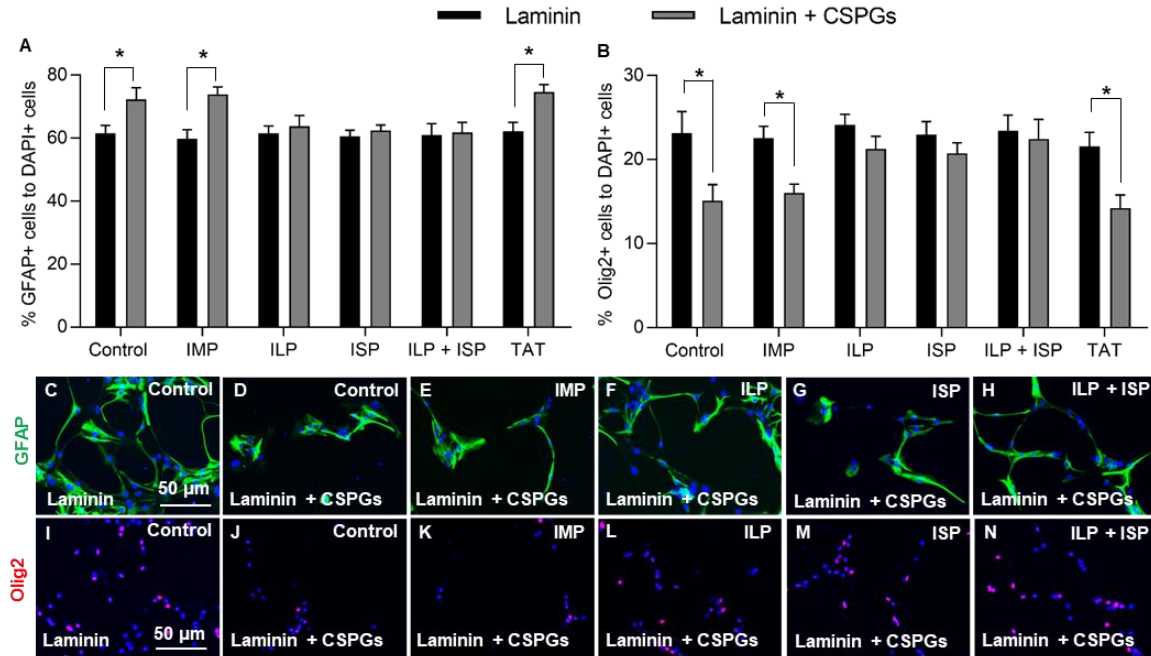
Spinal cord NPCs were grown on laminin (5μg/ml) or laminin (5μg/ml) + CSPGs (5μg/ml) substrate for 7 days in 2% FBS differentiation medium. The percentage of GFAP+/DAPI+ astrocytes (A) and Olig2+/DAPI+ oligodendrocytes (B) to the total number of DAPI+ NPCs was quantified. NPCs grown on laminin + CSPGs substrate showed a significant increase in astrocytes in comparison to laminin substrate alone. ILP, ISP or their combination blocked CSPG effects on astrocyte differentiation. (B) Exposure to CSPGs resulted in a significant decrease in the percentage of oligodendrocytes compared to laminin only control condition. ILP, ISP or ILP/ISP promoted the oligodendrocyte differentiation of NPCs on CSPGs substrate. (C-N) Representative images for NPCs differentiation assay are shown. N = 3 independent experiments. The data show the mean ± SEM. * P<0.05, One Way ANOVA.

3.4.1.3 ILP/ISP mitigate CSPGs induced dephosphorylation of Erk1/2 and AKT in NPCs: We also examined the potential of ILP and ISP to modulate downstream intracellular pathways involved in CSPGs/LAR and CSPGs/PTPσ signaling. Our previous work identified that CSPGs mediate their effects on NPCs by dephosphorylation of Erk1/2 and Akt pathways downstream of both LAR and PTPσ (Dyck et al. 2015). Here, we studied phosphorylation of Erk1/2 and Akt in NPCs under ILP/ISP treatment as a proof-of-principle for the efficacy of the peptides in attenuating LAR and PTPσ signaling activity. We found a significant 64% and 76% decrease in phosphorylation of both Erk1/2 (Supplemental Fig. 3.3A) and Akt (Supplemental Fig. 3.3B), respectively, in NPCs when exposed to a CSPGs substrate as anticipated based on our previous studies. Similar to our previous genetic studies (Dyck et al. 2015), we found that while dephosphorylation of Erk1/2 and Akt was non-significantly attenuated by ILP or ISP solitary treatment, only their combinatorial treatment was sufficient to significantly promote Erk1/2 phosphorylation in NPCs on a CSPGs substrate (N = 3, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc)*. Collectively, our *in vitro* assessments in NPCs show that co-inhibition of LAR and PTPσ by ILP/ISP can effectively block the inhibitory effects of CSPGs on all parameters of NPCs properties.

3.4.2 CSPGs negatively impact OPCs by signaling through LAR and PTPσ receptors and activation of the Rho/ROCK pathway

OPCs also play a critical role in oligodendrogenesis and remyelination after SCI (Hesp et al. 2015; Sun et al. 2010; McTigue and Tripathi 2008; Barnabe-Heider et al. 2010; Meletis, Barnabe-Heider, et al. 2008; Karimi-Abdolrezaee et al. 2012). Therefore, we additionally investigated the role of LAR and PTPσ in modulating the ability of OPCs for proliferation and oligodendrocyte maturation

in a CSPG environment *in vitro.* Understanding the role of CSPGs and their signaling receptors on both populations of NPCs and OPCs is critical to uncovering endogenous mechanisms of oligodendrogenesis after SCI. Previous studies have revealed that CSPGs limit OPCs process outgrowth and myelination *in vitro* (Pendleton et al. 2013; Lau et al. 2012; Larsen et al. 2003; Kuhlmann et al. 2008; Siebert, Stelzner, and Osterhout 2011). Here, utilizing ILP/ISP we investigated the involvement of LAR and PTPσ in mediating CSPG effects on various properties of OPCs, including their attachment, survival, proliferation and maturation. Cortical rat OPCs were plated onto growth substrates containing laminin or laminin and CSPGs. First, stereology-based measurements showed that presence of CSPGs significantly reduced OPCs attachment and their ability to spread their processes by 60.7% and 65.4%, respectively, as compared to laminin control condition. ILP and ISP treatment reversed CSPGs negative effects in a dose-dependent manner (Supplementary Fig. 3.2, N = 3, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). We identified a dose of 2.5μM to be optimal in blocking CSPG effects on OPCs. Using this optimal dose, we found that while ILP and ISP solitary treatment was able to block much of CSPG effects on OPCs attachment (Fig. 3.4A) and cell spreading (Fig. 3.4B-J), the combination of ILP and ISP was more effective in attenuating CSPGs inhibitory effects (N = 3, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Neither IMP (2.5μM) nor TAT (2.5μM) control peptides changed CSPG effects on OPCs attachment and process outgrowth demonstrating the specificity of ILP and ISP treatment. We additionally confirmed the specificity of CSPG effects by their degradation using ChABC. Interestingly, similar to NPCs, we found that inhibition of Rho/ROCK pathway in OPCs with Y-27632 significantly mitigates the inhibitory effects of CSPGs on OPCs. These data indicate the Rho/RCOK pathway as a common mechanism by which CSPGs regulate properties of NPCs and OPCs.

We next examined CSPG effects on OPCs proliferation and survival. Interestingly, our BrdU proliferation assay showed that in contrast to NPCs, CSPGs had no apparent modulatory effect on OPC proliferation *in vitro*. In addition, OPCs proliferation was unaffected by ILP, ISP, ILP + ISP, IMP, TAT or Y27632. However, similar to NPCs, our LIVE/DEAD assay revealed a significant 48.5% reduction in OPC survival when they were exposed to CSPGs substrate as compared to laminin control condition (Fig. 3.5B-T, N = 3, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). This effect was overcome by ILP, ISP, ILP + ISP, Y-2732 and ChABC, but not by IMP or TAT control peptides confirming specificity of CSPG effects that is mediated through LAR/PTPσ and activation of Rho/ROCK pathway. We extended our survival assessment to determine whether CSPGs drive cleaved caspase 3 mediated cell death in OPCs. The presence of CSPGs resulted in a significant 3.2-fold increase in the percentage of cleaved caspase 3 positive OPCs, which was significantly overcome by ILP, ISP, Y2732 and ChABC treatment. Importantly, co-treatment of ILP and ISP was able to more significantly block CSPG effects on caspase 3 mediated cell death in OPCs suggesting an important role for both LAR and PTPσin this mechanism (Fig. 3.5U-X, N=3, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*).

Lastly, we examined whether CSPGs modulate maturation of OPCs to oligodendrocytes. OPCs were grown onto laminin or laminin + CSPGs and allowed to mature for 10 days. We performed co-immunostaining for O4 and MBP, and considered cells with co-expression of O4 and MBP to represent mature oligodendrocyte phenotype. Our cell quantification showed that the presence of CSPGs inhibits maturation of OPCs to oligodendrocytes evidenced by a significant 32.0% reduction in the percentage of O4+/MBP+ cells compared to laminin control (Fig. 3.6A-M, N = 5, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Negative effect of CSPGs was partially overcome by ILP, ISP or Y-27632 but not by IMP or TAT treatment. Combinatorial

treatment of ILP+ISP was able to more effectively reverse the detrimental effects of CSPG effects on OPC maturation. ChABC treatment was able to overcome nearly all of CSPG effects on OPCs maturation.

Figure 3.4. CSPGs limit OPCs attachment and cell spreading which is overcome by ILP and ISP treatment and inhibition of the Rho/ROCK pathway.
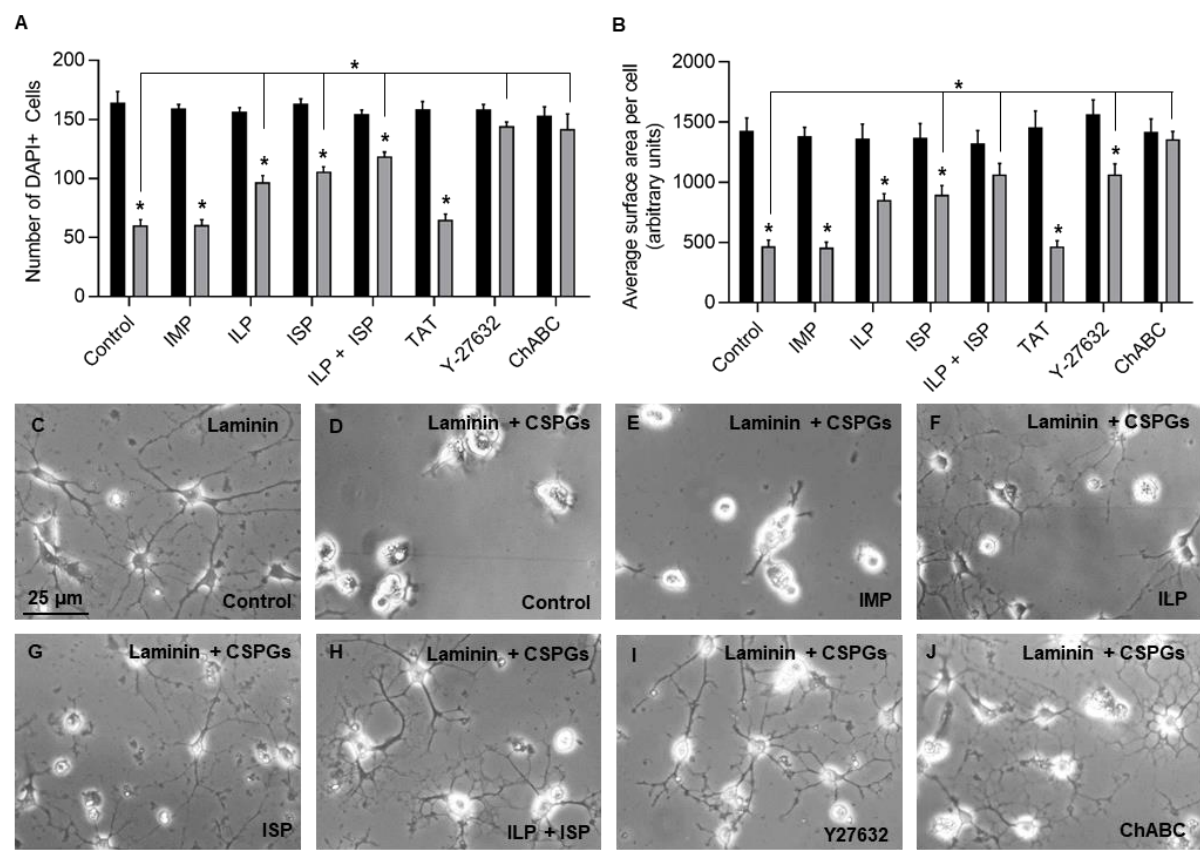
Figure 3.4. CSPGs limit OPCs attachment and cell spreading which is overcome by ILP and ISP treatment and inhibition of the Rho/ROCK pathway.

OPCs cell spreading and attachment was assessed by measuring the total number of DAPI+ cells (A) and the total cell surface area (B). Laminin + CSPGs substrate (D) significantly limited the ability of OPCs to attach and spread their cell processes compared to our laminin control (C) condition. ILP (F) and ISP (G) treatment promoted OPC attachment and cell spreading on CSPGs substrate which was significantly greater than laminin + CSPGs experimental group. (H) The combinatorial use of ILP and ISP further promoted OPC attachment and cell spreading on CSPGs substrate. (I) Inhibition of ROCK with Y-27632 was also sufficient to promote OPC attachment and cell spreading on CSPGs substrate suggesting CSPGs mediate their inhibitory effect on OPCs through activation of the Rho/ROCK pathway. (J) Degradation of CSPGs with ChABC confirmed CSPGs specificity to inhibiting OPC attachment and cell spreading. Control peptides IMP (E) and TAT had no apparent effect in blocking CSPG effects on OPCs. N = 3 independent experiments. The data show the mean ± SEM. * P<0.05, One Way ANOVA.

Figure 3.5. CSPGs limit OPCs survival and promote cleaved caspase 3 mediated apoptosis by signaling through LAR and PTPσ and activation of the Rho/ROCK pathway.
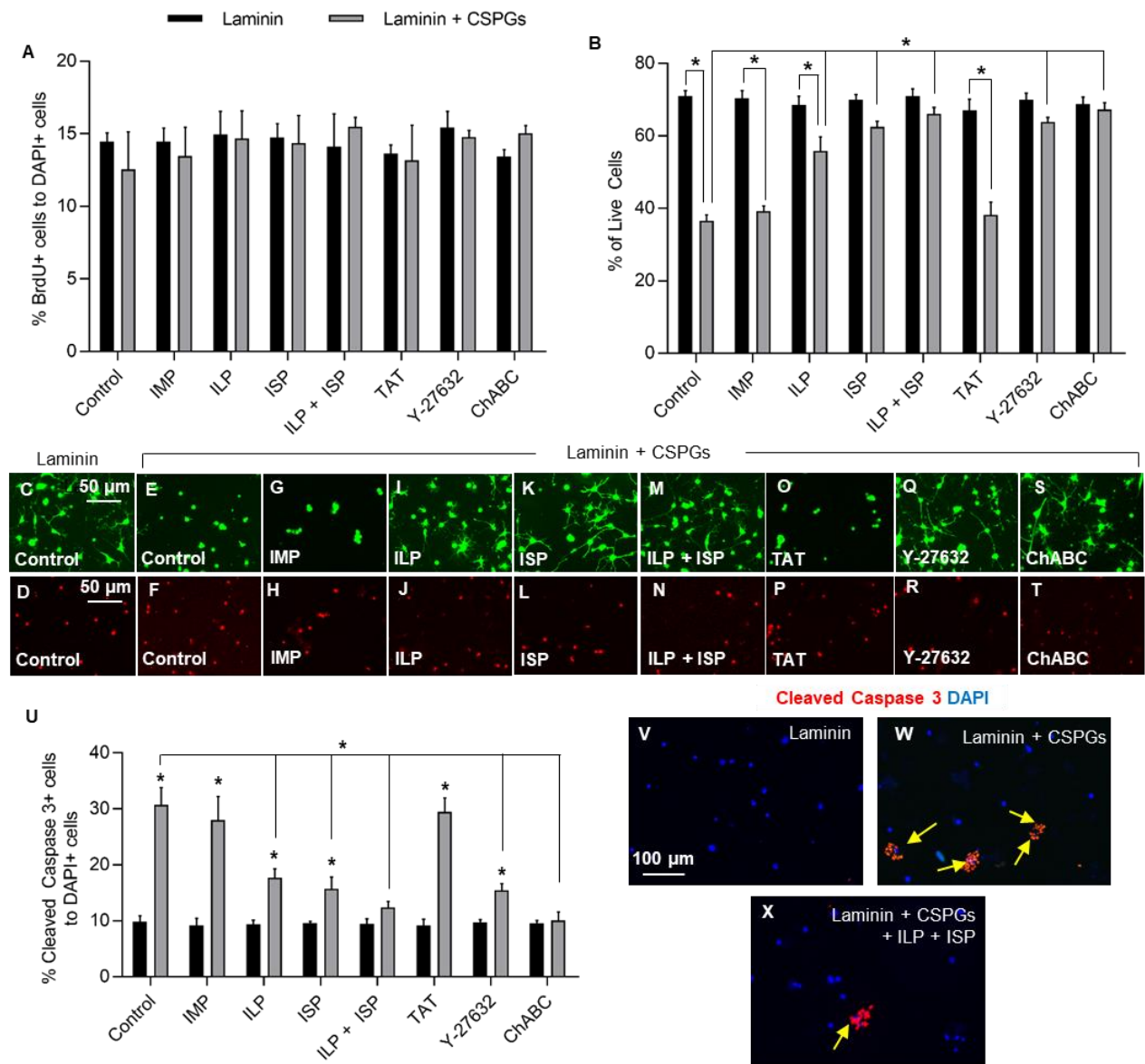
Figure 3.5. CSPGs limit OPCs survival and promote cleaved caspase 3 mediated apoptosis by signaling through LAR and PTPσ and activation of the Rho/ROCK pathway.

OPCs were grown on laminin or laminin + CSPGs substrate. (A) Using quantitative immunocytochemistry, we measured the percentage of BrdU+ cells to the total number of DAPI+ cells. Exposure to CSPGs or our various experimental groups had no apparent effect in modulating OPCs proliferation. (B-T) OPC survival on CSPGs substrate was assessed using a LIVE/DEAD assay. CSPGs (E-F) significantly limited OPCs survival compared to our laminin control (C-D) which was overcome by ILP (I-J) and ISP (K-L) treatment but not by control peptides IMP (G-H) or TAT (O-P). Combination of ILP and ISP (M-N) was able to further promote OPCs survival on CSPGs substrate. In addition, inhibition of the Rho/ROCK pathway with Y-27632 (Q-R) blocked the majority of CSPG effects on OPC survival. Degradation of CSPGs with ChABC (S-T) blocked all of CSPG effects on OPC survival confirming CSPGs specificity. (U-X) In addition, CSPGs were significantly increase the percentage of cleaved caspase 3 positive cells which was significantly overcome by ILP, ISP, ILP + ISP, Y-27632, and ChABC treatment but not by IMP or TAT. N = 3 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

Figure 3.6. CSPGs negatively modulate OPCs maturation *in vitro* which is overcome by inhibition of LAR and PTPσ and the Rho/ROCK pathway.
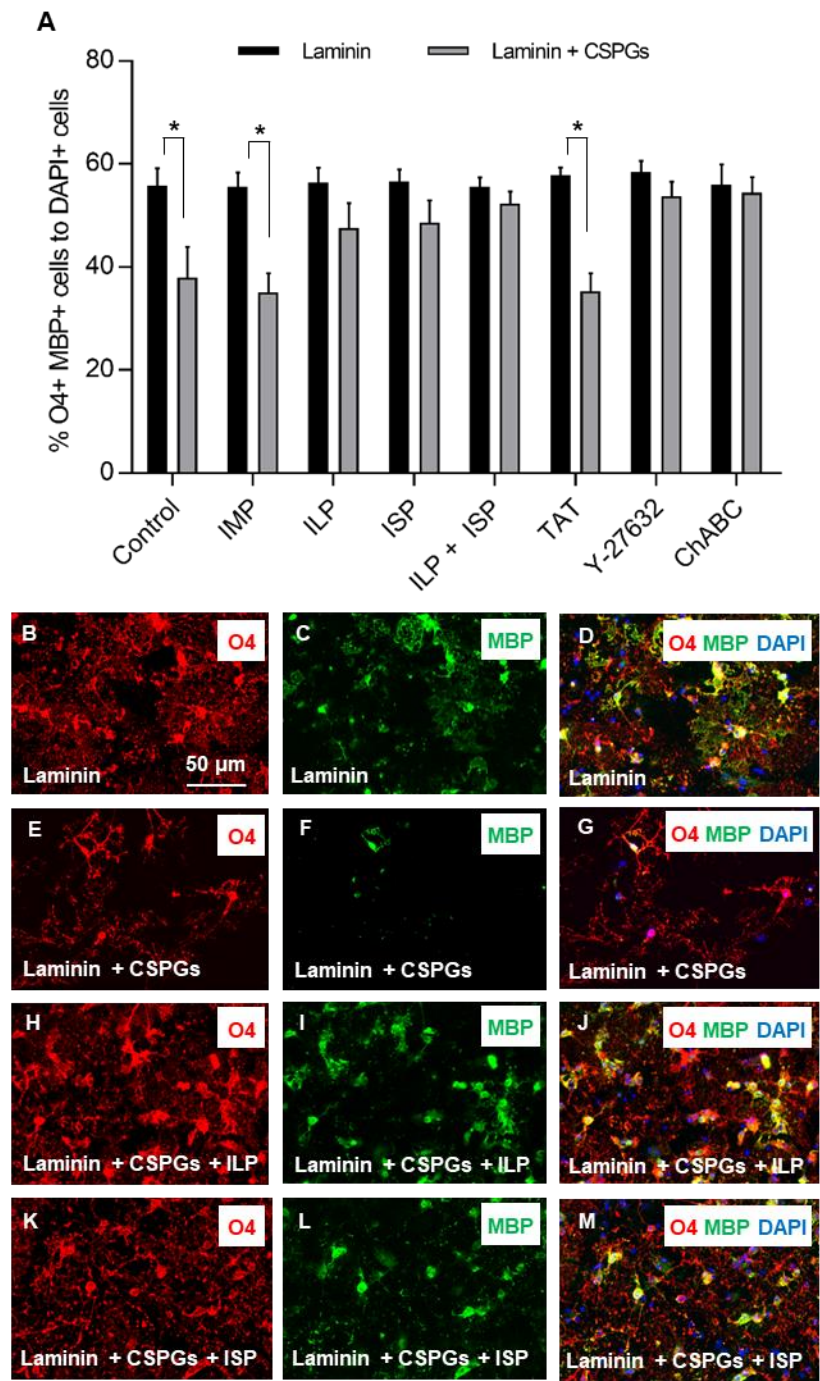
Figure 3.6. CSPGs negatively modulate OPCs maturation *in vitro* which is overcome by inhibition of LAR and PTPσ and the Rho/ROCK pathway.

OPCs were grown on laminin or laminin + CSPGs substrate for 10 days and then assessed for their maturation. (A) Using quantitative immunocytochemistry, we measured the percentage of O4+ MBP+ cells. (B-M) The presence of CSPGs significantly reduced the percentage of O4+ MBP+ cells compared to laminin control. This was partially overcome by both ILP and ISP (E) treatment, with the combination of ILP and ISP further limiting CSPG effects. Inhibition of the Rho/ROCK pathway with Y-27632 was able to block much of CSPG effects. Degradation of CSPGs with ChABC blocked nearly all of CSPG effects on OPCs maturation confirming CSPGs specificity. Both control peptides, IMP and TAT, had no apparent effect on blocking CSPG effects. N = 5 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

3.4.3 ILP and ISP Promote Endogenous Cell Proliferation and Oligodendrogenesis after SCI

Based on our *in vitro* findings, we next utilized ILP and ISP to determine the role of LAR and PTPσ in oligodendrogenesis in a clinically-relevant model of compressive/contusive SCI in rats. We delivered TAT-conjugated ILP and ISP treatments intrathecally to the area of SCI in a sustainable manner using an Alzet osmotic pump. In our previous SCI work, we confirmed the ability of TAT-conjugated ILP and ISP peptides to enter the spinal cord tissue successfully (Lang et al. 2015). We first conducted a dosing study to determine the optimal intrathecal concentration of ILP/ISP in modulation of the endogenous oligodendrogenesis after SCI. We began infusion of ILP/ISP intrathecally at the time of SCI at doses of 5μg, 10μg, and 15μg per day for duration of either 7 days (N=4 animals/group) or 28 days post-SCI (N=5-6 animals/group), representing subacute and early chronic phases of SCI, respectively (Table 3.1). ILP and ISP were co-delivered as our *in vitro* data identified that both LAR and PTPσ were involved in modulating CSPG effects on NPCs and OPCs, and that ILP/ISP co-treatment had greater effects than their solitary administration. Functional stability of ILP and ISP in Alzet osmotic pumps was verified using our *in vitro* attachment assay confirming that ILP and ISP were stable up to 42 days (Supplemental Fig. 3.4). We labeled newly generated cells in the injured spinal cord during the treatment period through BrdU pulsing starting at the time of SCI (Fig. 3.7A-E). At the end of 7-day and 28-day treatment strategy, we studied the outcomes of ILP/ISP treatment on endogenous cell proliferation and differentiation in the injured spinal cord. We performed phenotypic analysis of BrdU labeled cells in 6 specific regions in spinal cord cross sections including ependymal/subependymal region as well as dorsal, lateral and white matter columns (Fig. 3.7C). Our cellular assessments were performed at 1-2mm rostral point to the injury epicentre for the 7-day early subacute time-point and at 2-3mm rostral to the injury site for the 28-day early chronic time-point. These distances

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were selected to allow inclusion of the injury penumbra that contained intact ependymal/subependymal regions where NPCs reside in the spinal cord for each time-point.

At 7 days post-SCI, quantification of BrdU labeled cells showed a non-significant 37% and 38% increase in the total number of BrdU+ cells with daily 10µg and 15µg of ILP/ISP treatment, respectively, compared to vehicle SCI groups (Fig. 3.7A, $p > 0.05$, One Way ANOVA). In the 28-day study, we found a significant 46% and 42% increase in the number of newly generated BrdU+ cells, respectively, in the group that received ILP/ISP (10µg/day) compared to vehicle treated rats (Fig. 3.7B-E, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Our phenotypic analysis of these BrdU+ cells at 7 days post-SCI showed a significant 16-20% decrease in the percentage of new astrocytes (GFAP+/BrdU+) at 1mm and 2mm rostral to the injury site in the 10µg and 15µg ILP/ISP treatment groups (Fig. 3.8A, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). This effect was further pronounced in our 28-day study where we observed a 28-32% decrease in the percentage of BrdU+ astrocytes (Fig. 3.8B, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Importantly, ILP/ISP treatment increased the generation of new oligodendrocytes (Olig2+/BrdU+). In both 7 and 28 days post-injury endpoints, we found a significant 60-72% and 35-58% increase, respectively, in the number of new Olig2+/BrdU+ new oligodendrocytes for the 10µg and 15µg ILP/ISP treated animals compared to vehicle controls (Fig. 3.8C-D, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). This effect was more prominent at distances further from the injury site. More importantly, we confirmed that these newly formed oligodendrocytes were differentiating into mature oligodendrocytes by co-labelling BrdU and APC (a mature oligodendrocyte marker) in the 28-day chronic SCI animals at 2mm rostral to the injury site (Fig. 3.8G-H, $p < 0.05$, Student *t* test). Overall, we observed a significant 66% increase in the percentage of BrdU+/APC+ cells in our 10µg/day ILP/ISP treated group. Of note, in our *in vivo* assessment

newly generated oligodendrocytes are attributed to progenies of both NPCs and OPCs as these two populations cannot be differentiated in our rat SCI model. Taken together, these findings indicate that ILP and ISP co-treatment at 10µg/day dosage promotes endogenous oligodendrogenesis following SCI.

Figure 3.7. ILP and ISP co-treatment promotes endogenous cell proliferation following SCI.
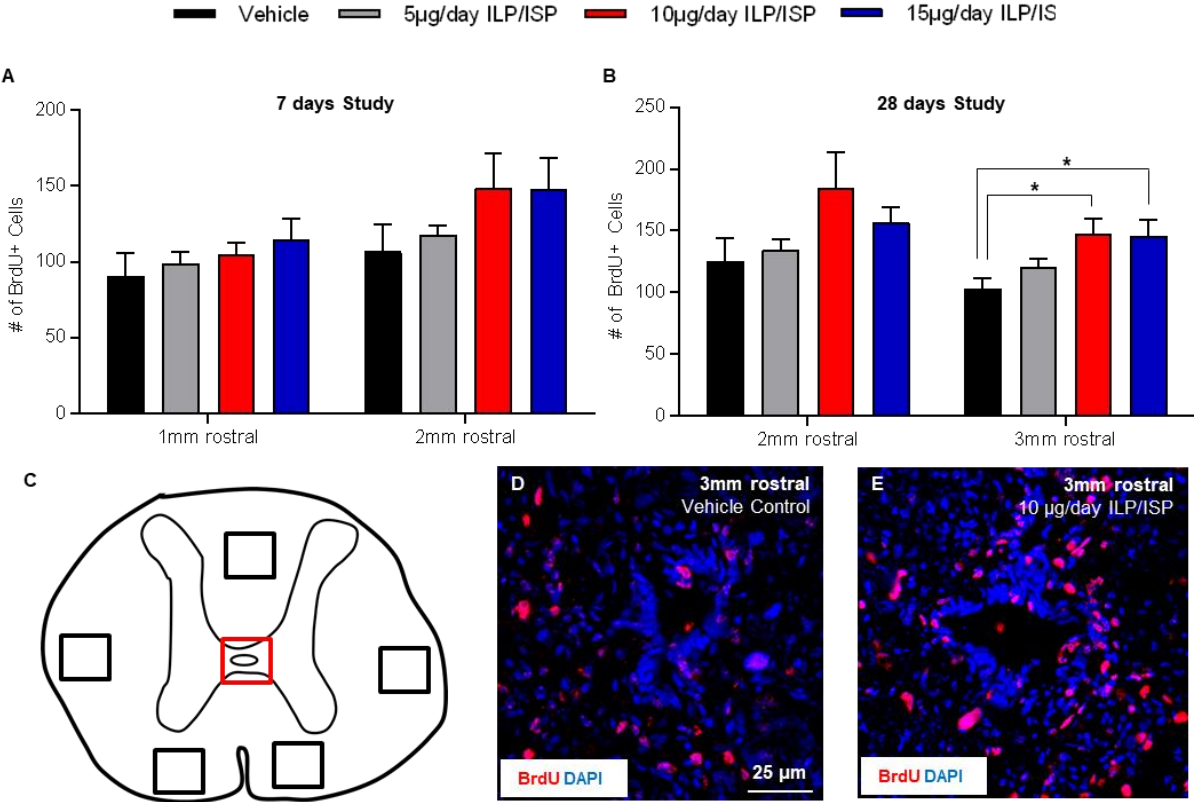
Figure 3.7. ILP and ISP co-treatment promotes endogenous cell proliferation following SCI.

(A-B) Our dosing experiments for ILP/ISP co-treatment in rat SCI showed an overall increase in the number of BrdU labelled cells in the spinal cord of the group that received a daily dose of 10µg and 15µg ILP/ISP for 7 and 28 days post-SCI. This increase was significant for both 10µg/day and 15µg/day ISP + ILP treated group at 28 days. (C) Quantification of BrdU labelled cells was conducted in specific regions of spinal cord cross sections including the ependymal/subependymal region, dorsal and lateral columns, and ventral white matter regions. (D-E) Representative cross sections of the injured spinal cord in the ependymal/subependymal region labelled for BrdU (new cells) and DAPI (nuclear marker) at 28 days post-SCI show that ILP/ISP treatment (10µg/day) enhanced cell proliferation in the ependymal and peri-ependymal areas at 3 mm distance to the injury center. N = 4-6 animals/group. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

Figure 3.8. ILP and ISP co-treatment promote oligodendrogenesis after SCI.
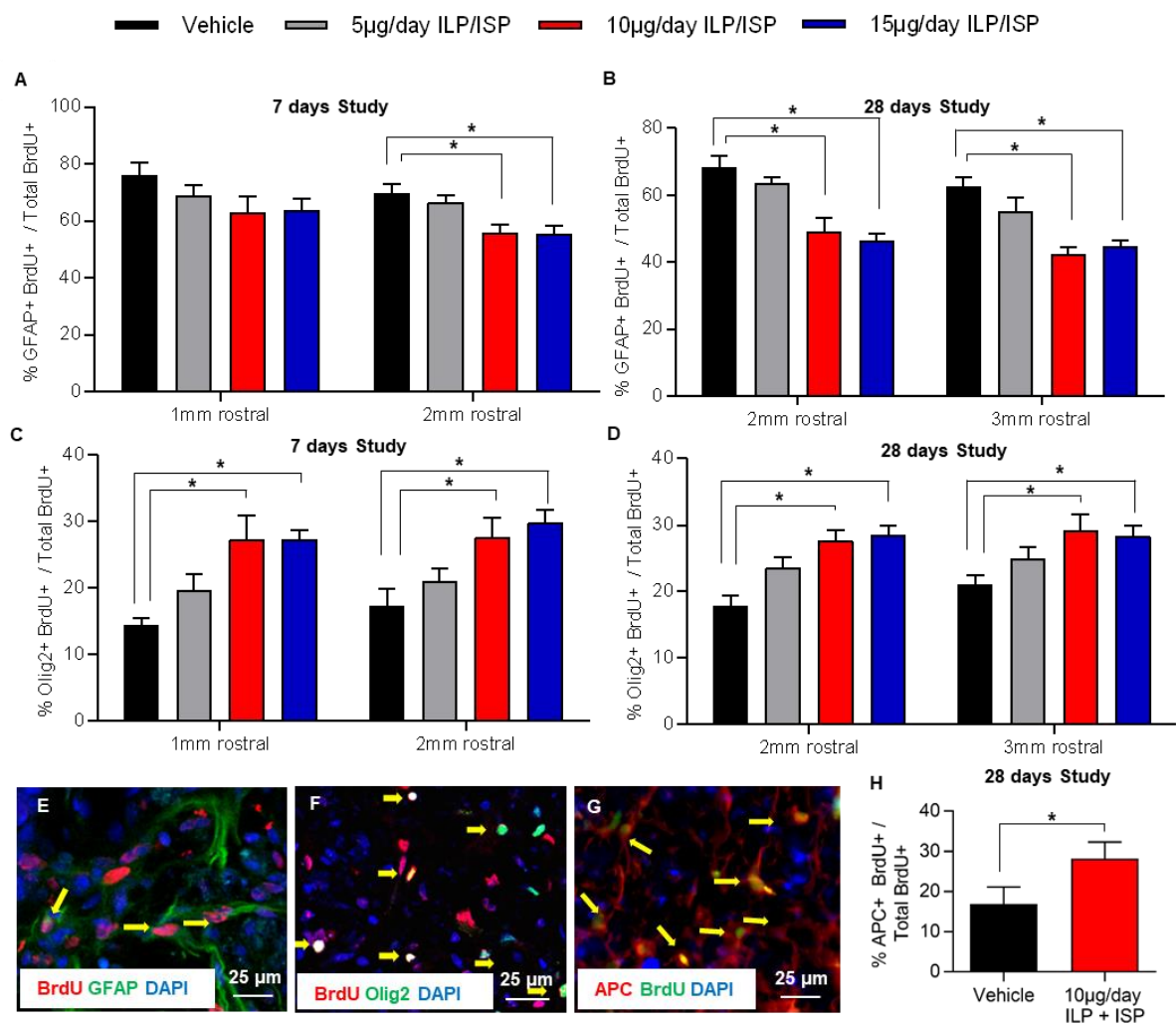
Figure 3.8. ILP and ISP co-treatment promote oligodendrogenesis after SCI.

(A-B, E) Phenotypic analysis of newly-generated BrdU+ cells at 7 and 28 days following ILP/ISP treatment showed a significant decrease in the number of newly formed astrocytes (BrdU+/GFAP+) with 10µg/day and 15µg/day of ILP/ISP compared to the 5µg/day and vehicle treated groups at the 2 and 3mm rostral points to the injury center. (C-D, F) Conversely, there was a significant increase in the number of newly formed cells within the oligodendrocyte lineage (BrdU+/Olig2+) under the same treatment and at the same time-point and distances. (G-H) Additionally, we observed a significant number of new oligodendrocytes acquired a mature phenotype (APC+/BrdU+) under 10µg/day ILP/ISP treatment at 28 days following injury. N = 4-6 animals/group. The data show the mean ± SEM, *$p<0.05$, One Way ANOVA, except for H which was Student *t*-test,

3.4.4 Inhibition of LAR and PTPσ Confers Protection to Mature Oligodendrocytes by Attenuating Caspase-3 Mediated Apoptosis Following SCI

After SCI, mature oligodendrocytes undergo significant loss in their population due to their susceptibility to apoptosis (Beattie 2004; Robins-Steele, Nguyen, and Fehlings 2012; Casha, Yu, and Fehlings 2001). Our tissue assessment of oligodendrocyte population at 7 and 28 days post-SCI showed a higher percentage of oligodendrocytes identified by the lineage marker, Olig2, in 10μg and 15μg ILP/ISP treatment groups at 1mm and 2mm rostral to the injury site compared to the vehicle treated SCI group (Fig. 3.9A-F, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Moreover, our analysis of at 7 days post-treatment showed a significant 42% and 51% increase in preservation of mature oligodendrocytes (APC+) at 1mm and 2mm rostral to the injury site, respectively, in the 10μg/day of ILP/ISP treated rats compared to vehicle treated group (Fig. 3.10A, $p < 0.05$, Two Way ANOVA, Holm-Sidak *post hoc*). Furthermore, we found a significant 1.5-fold increase in the immunointensity of MBP in the white matter regions at 1, 2, and 3mm rostral to the injury site in ILP/ISP treated animals compared to vehicle control (Fig. 3.10B-D, $p < 0.05$, Two Way ANOVA, Holm–Sidak *post hoc*).

Given the overall increase in preservation of mature oligodendrocytes and myelin, we next sought to determine whether ILP/ISP treatment attenuates oligodendrocytes apoptosis following injury. We first studied the overall protein expression of cleaved caspase-3 as a mediator of apoptosis in SCI at 1, 3, and 7 days post treatment using Western blotting (Fig. 3.10E). While there was no apparent change at 1 day, ILP/ISP treated (10μg/day) rats showed a significant 48.5% and 30.8% decrease in cleaved caspase-3 protein expression at both 3 and 7 days post treatment, respectively, compared to the SCI vehicle group ($p < 0.05$, One Way ANOVA, Holm-Sidak *post hoc*). Moreover, we specifically assessed apoptosis in mature oligodendrocytes by co-

immunolabeling between APC and cleaved caspase 3 at 7 days post-injury (Fig. 3.10F-I). ILP/ISP treatment significantly decreased the number of apoptotic oligodendrocytes at 2mm rostral point to the injury site (Fig. 3.10F, One Way ANOVA, Holm-Sidak *post hoc*). Altogether, these data indicate that activation of LAR and PTPσ in the injured spinal cord contributes to oligodendrocyte apoptosis and myelin degeneration that can be attenuated by ILP and ISP treatment.

Our *in vitro* findings unraveled an involvement for the Rho/ROCK as a downstream mechanism in mediating the effects of CSPGs signaling on cell death in NPCs and OPCs. Thereby, we next investigated whether ILP/ISP can attenuate RhoA activation in SCI. Using G-LISA, we assessed RhoA activity by examining the amount of GTP-bound Rho (Kanazawa et al. 2013) . At 3 days post SCI, we observed a significant 10.6-fold increase in RhoA activity, which was significantly blocked by 10μg/day ILP/ISP treatment (Fig, 3.10J, N = 4, One Way ANOVA, Holm-Sidak *post hoc*). Our data provides the first evidence suggesting an association between LAR and PTPσ receptors and RhoA signaling in regulating oligodendrocytes survival and replacement following SCI.

Figure 3.9. ILP and ISP promotes oligodendrocyte preservation after SCI.
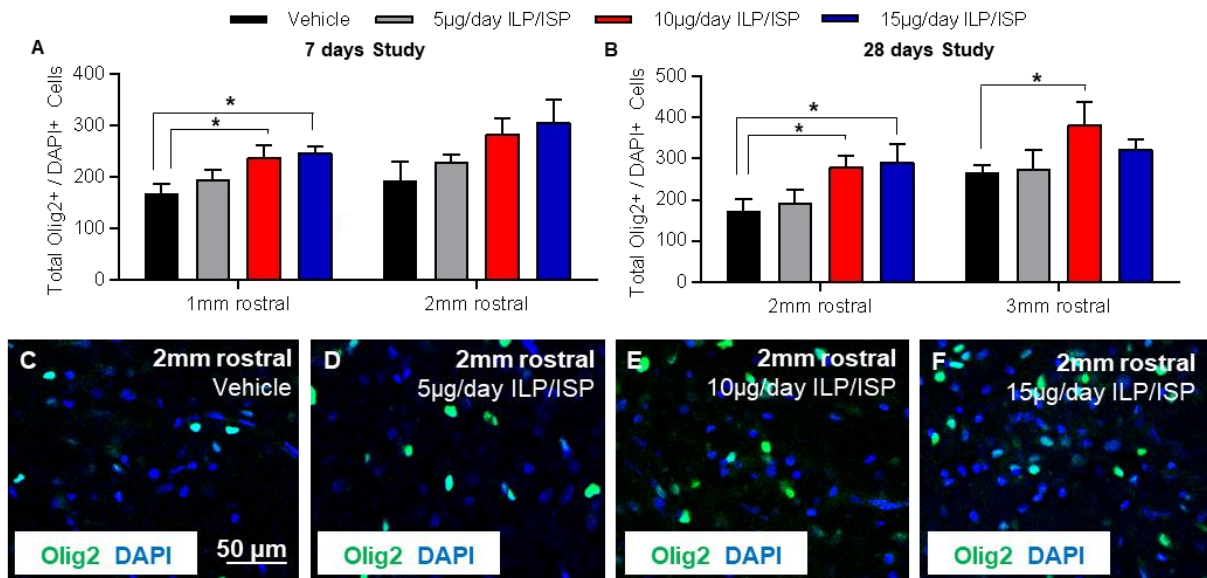
(A-F) We assessed the number of oligodendrocytes in the spinal cord at 7 and 28 days post-SCI. Treatment with ILP and ISP (10μg/day and 15μg/day) resulted in a remarkable increase in the number of Olig2+ oligodendrocytes at 7 (A) and 28 (B) days post-injury. N = 4-6 animals/group. The data show the mean ± SEM. *p<0.05, One Way ANOVA.

Figure 3.10. Inhibition of LAR and PTPσ attenuates caspase-3 mediated apoptosis in mature oligodendrocyte following SCI.
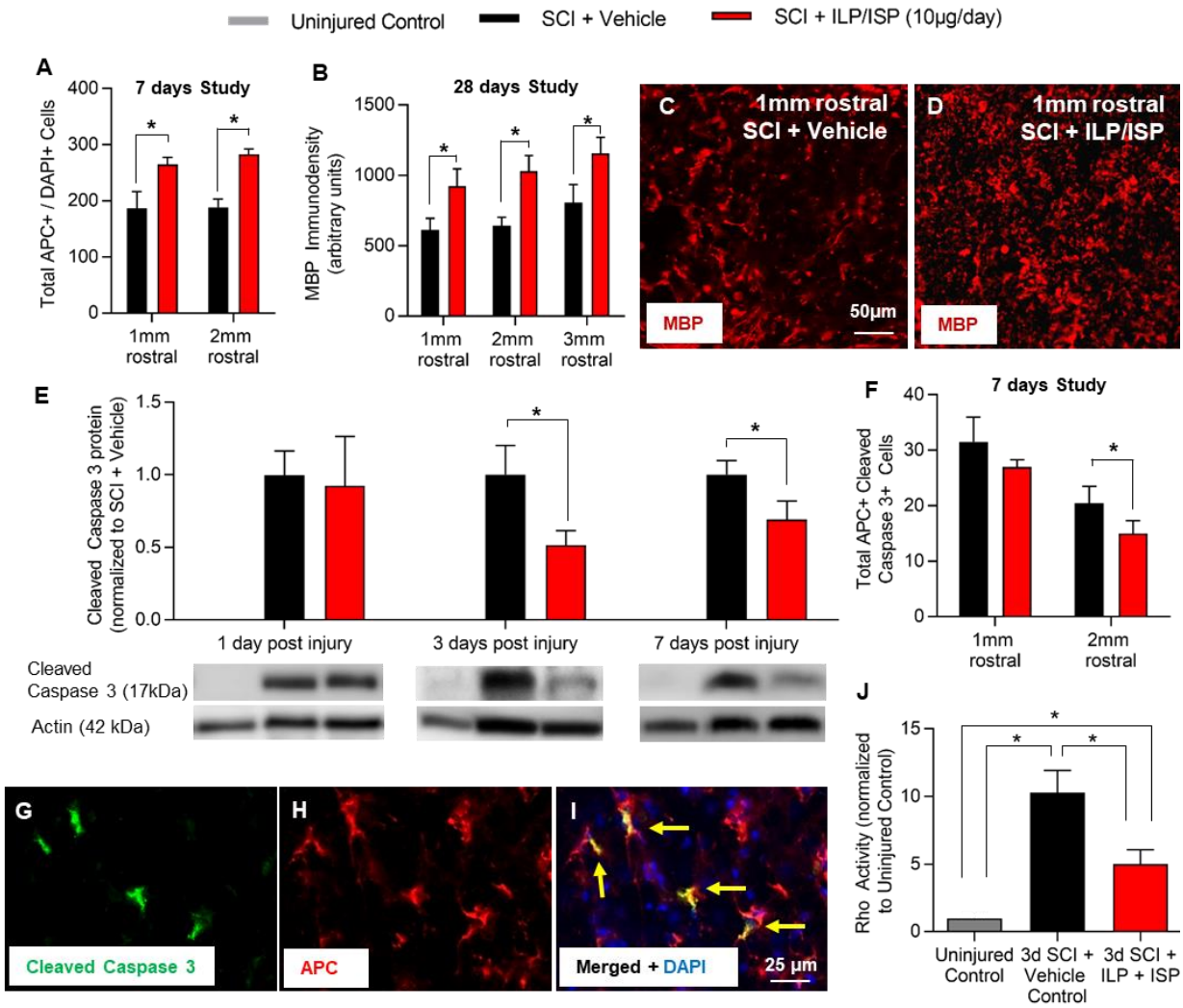
Figure 3.10. Inhibition of LAR and PTPσ attenuates caspase-3 mediated apoptosis in mature oligodendrocyte following SCI.

(A) Preservation of mature APC+ oligodendrocytes was assessed at 7 days following injury when apoptosis is normally underway. There was a significantly higher number of APC+ oligodendrocytes under ILP and ISP treatment at 1mm and 2mm rostral points to the injury. (B-D) Immunodensity of myelin basic protein (MBP, a signature marker for myelin) at 28-day post-injury in the white matter regions at 1, 2, and 3mm rostral to the injury site showed a significant increase in MBP expression in ILP/ISP treated SCI animals compared to SCI vehicle control. (E) Western blotting showed an overall reduction in cleaved caspase-3 expression at 1, 3 and 7 days post-injury under 10μg/day of ILP and ISP, which was significantly different at 3 and 7 days post-injury compared to vehicle group. (F-I) Co-immunohistochemistry for APC and cleaved caspase 3 at 7 days post-SCI revealed a significant reduction in number of apoptotic oligodendrocytes at 2mm rostral point to the injury in the spinal cord white matter of rats treated with ILP/ISP compared to the rats treated with vehicle. (J) Rho activity was assessed by G-LISA at 3 days post SCI. We found a significant increase in Rho activity at 3 days post-injury which was significantly reduced by ILP and ISP treatment. N = 4-6 animals/group. The data show the mean ± SEM. Cleaved caspase-3 results have been normalized to the actin loading control prior to subsequent normalization to the SCI + Vehicle values, *p<0.05, Two Way ANOVA (A-B, F), One Way ANOVA (E, J).

3.5 Discussion

In this study, we have uncovered a novel inhibitory role for LAR and PTPσ in endogenous regulation of oligodendrocytes in the injured adult spinal cord. Utilizing specific functionally blocking peptides, ILP/ISP, in SCI-relevant models, we demonstrate that inhibition of LAR and PTPσ promotes oligodendrocyte differentiation and maturation. Moreover, ILP/ISP promoted oligodendrocyte and myelin preservation after SCI by mitigating cleaved caspase 3 mediated apoptosis in mature oligodendrocytes. Interestingly, our parallel *in vitro* and *in vivo* findings identified the RhoA/ROCK pathway as an underlying intracellular mediator for the inhibitory effects of CSPGs and LAR and PTPσ on oligodendrocyte survival and integrity. Thereby, we have uncovered, for the first time, new endogenous mechanisms by which CSPGs and LAR/PTPσ axis hinder repair processes and consequently recovery following SCI (Lang et al. 2015; Fisher et al. 2011).

SCI results in spontaneous activation of endogenous precursor cells, NPCs and OPCs; however, the ability of these cells to generate mature oligodendrocytes is limited in the injured spinal cord (Barnabe-Heider et al. 2010; Mothe et al. 2005; Meletis et al. 2008; Karimi-Abdolrezaee et al. 2012). Because oligodendrocyte population undergoes significant cell death following SCI, enhancing their preservation and replacement is a critical strategy for spinal cord repair (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015; Casha, Yu, and Fehlings 2001; Beattie et al. 2002). Our previous genetic studies *in vitro* unraveled that CSPGs directly impede the regenerative capacity of adult NPCs for oligodendrogenesis. Moreover, we identified the involvement of both LAR and PTPσ in mediating CSPGs inhibition of NPCs (Dyck et al. 2015). In the present study, utilizing ISP and ILP, we extended these initial discoveries to a clinically-relevant model of contusive/compressive SCI in rat to uncover the impact of LAR and PTPσ on

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the response of endogenous precursor cells and their ability for oligodendrocyte differentiation. Since oligodendrogenesis in the injured adult spinal cord is attributed to both populations of NPCs and OPCs, we also conducted parallel *in vitro* experiments on primary NPCs and OPCs. These *in vitro* studies were crucial in understanding the role of LAR and PTPσ in regulating NPCs and OPCs because in our rat SCI we are not able to specifically study the two population. Here, our parallel studies show that CSPGs negatively influences the regenerative abilities of both NPCs and OPCs populations through LAR and PTPσ mediated mechanisms. We find that blockade of each receptor individually by ILP or ISP was effective to partially reverse CSPG effects on NPCs and OPCs in culture. However, the co-inhibition of LAR and PTPσ with ILP and ISP maximized their solitary beneficial effects. As a result, we delivered ILP and ISP in combination in our SCI studies.

In rat SCI, for the first time, we demonstrate that co-blockade of LAR and PTPσ remarkably enhances oligodendrogenesis in the subependymal as well as in the white matter regions of the spinal cord where adult NPCs and OPCs reside, respectively. These beneficial effects were also accompanied by a reduction in the number of newly generated astrocytes. This is particularly important as it is known that the SCI niche instructs NPCs to give rise to astrocytes with only a limited number differentiating into oligodendrocytes (Barnabe-Heider et al. 2010; Meletis et al. 2008; Sabelström et al. 2013; Karimi-Abdolrezaee et al. 2010; Mothe and Tator 2005). Previous studies from our group in rat SCI showed that ChABC treatment can promote the survival and oligodendrocyte differentiation of transplanted NPCs (Karimi-Abdolrezaee et al. 2010). It is important to note that detection of newly generated oligodendrocytes by BrdU incorporation approaches does not allow us to discern whether oligodendrogenesis occurred through NPCs or OPCs. Therefore, the robust increase in endogenously generated oligodendrocytes that we observed following ILP and ISP treatment is likely attributed to modulation of both NPC and OPC

populations. In agreement with our *in vitro* findings in OPCs, CSPGs have previously been shown to limit process outgrowth of OPCs and their myelination by signaling through PTPσ (Pendleton et al. 2013; Lau et al. 2012). Taken together, our new findings show that dysregulated activation of CSPGs and LAR/PTPσ signaling in the injured spinal cord appears to be an underlying cause of inadequate oligodendrogenesis following SCI.

Oligodendrocyte population are highly susceptible to delayed apoptosis in SCI resulting in the loss of mature and newly generated oligodendrocytes as the injury evolves (Beattie, Farooqui, and Bresnahan 2000; Casha, Yu, and Fehlings 2001). It is established that loss of oligodendrocytes leads to demyelination and contributes to functional deficits following SCI (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Therefore, it is critical to uncover injury mechanisms that contribute to oligodendrocyte apoptosis in SCI. Here, we have identified a novel role for CSPGs in inducing caspase 3 mediated apoptosis in NPCs and OPCs. Importantly, we show that perturbing LAR and PTP□ signaling following SCI protects mature oligodendrocytes within their injury environment. Few studies have implicated CSPGs in modulating neuronal atrophy or death in SCI (Carter, McMahon, and Bradbury 2011; Carter et al. 2008), however to our knowledge; our study is the first that links CSPGs to oligodendrocyte apoptosis. Studies by Carter and colleagues showed that intrinsically labeled corticospinal neurons in layer V of the sensorimotor cortex undergo progressive atrophy, with shrinkage of cell bodies but no evidence of cell death at four weeks post injury in a dorsal column injury model of mouse SCI (Carter et al. 2008). Cellular atrophy was overcome by ChABC treatment (Carter et al. 2008) and was later shown to also occur in rubrospinal neurons in a C5 lateral column SCI model (Carter, McMahon, and Bradbury 2011). Interestingly, in a transection model of SCI in lamprey, high expression of PTPσ in neurons was directly correlated with higher expression of caspase activation indicating a role for PTPσ in the

retrograde neuronal death following SCI (Zhang et al. 2014). Altogether, our findings has provided novel evidence that CSPGs contribute to apoptosis in oligodendrocytes through LAR and PTPσ, and pose a challenge on their replacement by limiting endogenous oligodendrogenesis in SCI.

Intracellularly, we identified that Rho activation is a putative mechanism by which CSPGs signaling modulate cell death in NPCs, OPCs and mature oligodendrocytes. We and others previously showed activation of the Rho/ROCK pathway downstream to CSPGs signaling in NPCs (Dyck et al. 2015) and neurons (Dergham et al. 2002; Duffy et al. 2009; Monnier et al. 2003). In NPCs, we found that ROCK inhibition allows NPCs to grow on CSPGs and promote their survival and oligodendrogenesis (Dyck et al. 2015). More recently, both LAR and PTPσ have been implicated in mediating Rho activation in primary cerebellar granule neurons using genetic knockdown approaches (Ohtake et al. 2016). *In vivo*, Rho activation has also been associated with p75 mediated apoptosis in neurons and glial cells in rodent SCI (Dubreuil, Winton, and McKerracher 2003), however its link to LAR and PTPσ has not be reported. *In vitro,* we demonstrate that CSPGs directly promote cleaved caspase-3 mediated apoptosis in both NPCs and OPCs, which is driven through LAR and PTPσ and activation of the Rho/ROCK pathway. Notably, Rho activation has been temporally correlated with induced cell death after SCI, which can be overcome by Rho antagonists (Dubreuil, Winton, and McKerracher 2003; Eftekharpour et al. 2016). Our new findings *in vitro* and in rat SCI indicate that LAR and PTPσ appears to be involved in regulating Rho/ROCK pathway, and their inhibition with ILP/ISP is able to significantly attenuate the SCI induced increase in RhoA activity. Interestingly, our data also established a temporal correlation between the increase in RhoA activity and caspase 3 mediated cell death after SCI as both RhoA activity and cleaved caspase 3 activation were attenuated by ILP/ISP treatment.

Hence, our data suggest a Rho/ROCK mechanism in governing LAR and PTPσ mediated apoptosis in oligodendrocyte after SCI.

In conclusion, we provide novel evidence that CSPGs receptors, LAR and PTPσ, promotes oligodendrocyte apoptosis and myelin damage, and inhibits oligodendrocyte differentiation and maturation after SCI. Importantly, we have unraveled that CSPGs and LAR/PTPσ axis can negatively regulate both populations of NPCs and OPCs, and restrict their survival and their ability for oligodendrogenesis. We identified that LAR and PTPσ signaling appears to mediate their effects by RhoA activation. Thus, our work has uncovered a previously unknown role for LAR and PTPσ in SCI, and provides new insights into the endogenous mechanisms by which targeting CSPGs signaling has improved the outcomes of SCI in earlier studies in addition to facilitating axon regeneration and plasticity (Lang et al. 2015; Fisher et al. 2011). Therapeutically, our work also highlights the promise of ILP/ISP as a new feasible and effective treatment strategy for enhancing oligodendrocyte preservation and replacement after SCI, and other CNS conditions in which CSPGs impedes endogenous cell differentiation.

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3.7 Supplementary Materials

3.7.1 Supplementary Figures

Supplementary Figure 3.1: ILP and ISP overcome the inhibitory effects of CSPGs on NPC cell spreading and attachment in a dose dependant manner.
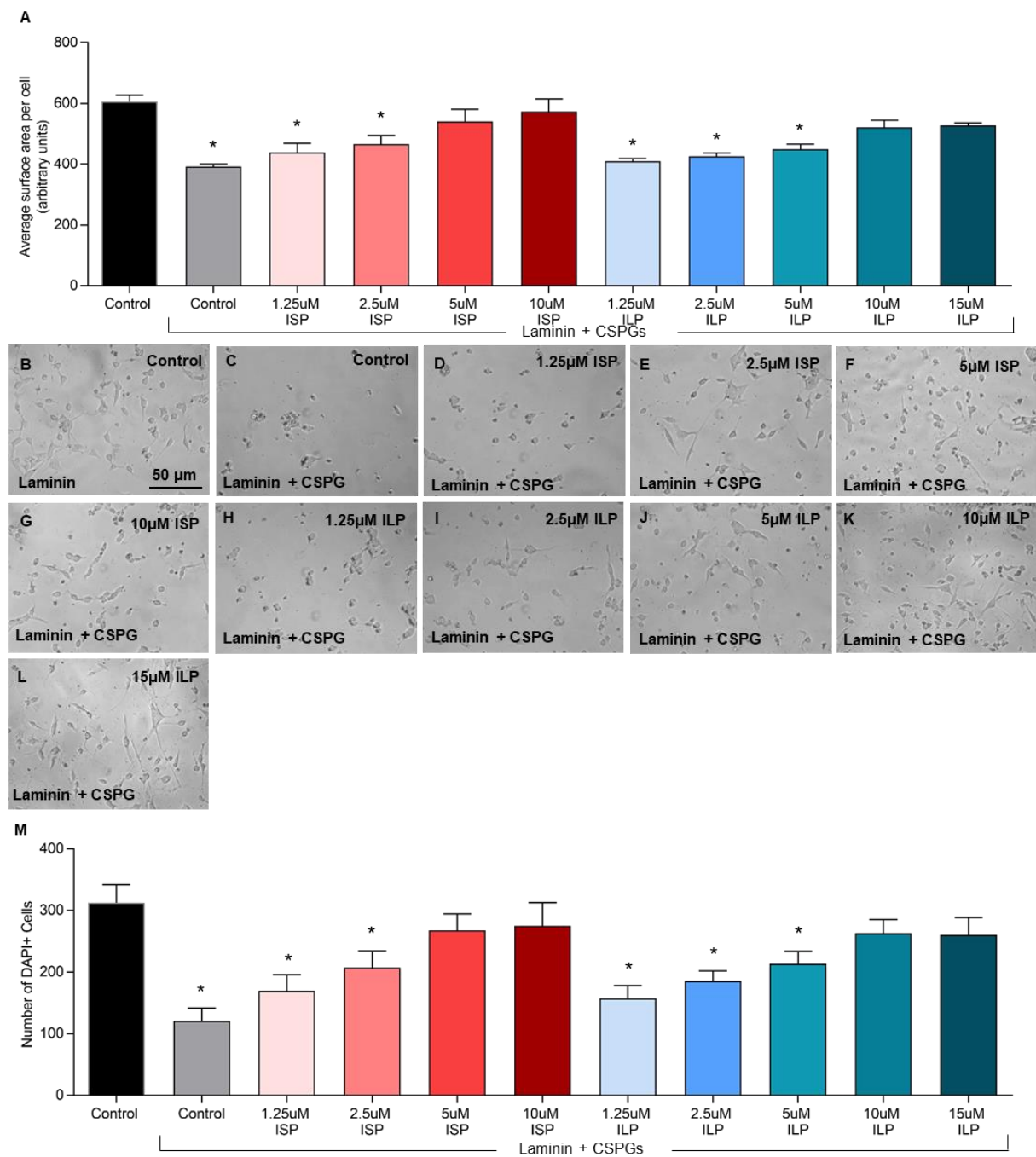
Supplementary Figure 3.1: ILP and ISP overcome the inhibitory effects of CSPGs on NPC cell spreading and attachment in a dose dependant manner.

Spinal cord NPCs were grown on laminin (5μg/ml) or laminin (5μg/ml) + CSPGs (5μg/ml) substrate to mimic the normal and post-SCI matrix composition, respectively. (A-L) Bright field images of spinal cord NPCs at 1 day after plating showed limited attachment and cell spreading on CSPGs (C) compared to laminin only (B). Inhibition of PTPσ (D-G) and LAR (H-L) with ISP and ILP, respectively, blocked CSPG effects on cell spreading in a concentration dependent manner. NPCs showed the greatest improvement in cell spreading when treated with 10μM ISP (G) and 10μM ILP (K). (M) Similarly, NPCs attachment was significantly decreased on CSPGs substrate that was partially stored with both ISP and ILP with the greatest effect seen by 10μM ISP (G) and 10μM ILP (K). N = 3 independent experiments. The data show the mean ± SEM. * P<0.05, One Way ANOVA.

Supplementary Figure 3.2: ILP and ISP limit CSPG effects on OPCs attachment and cell spreading in a dose dependant manner.
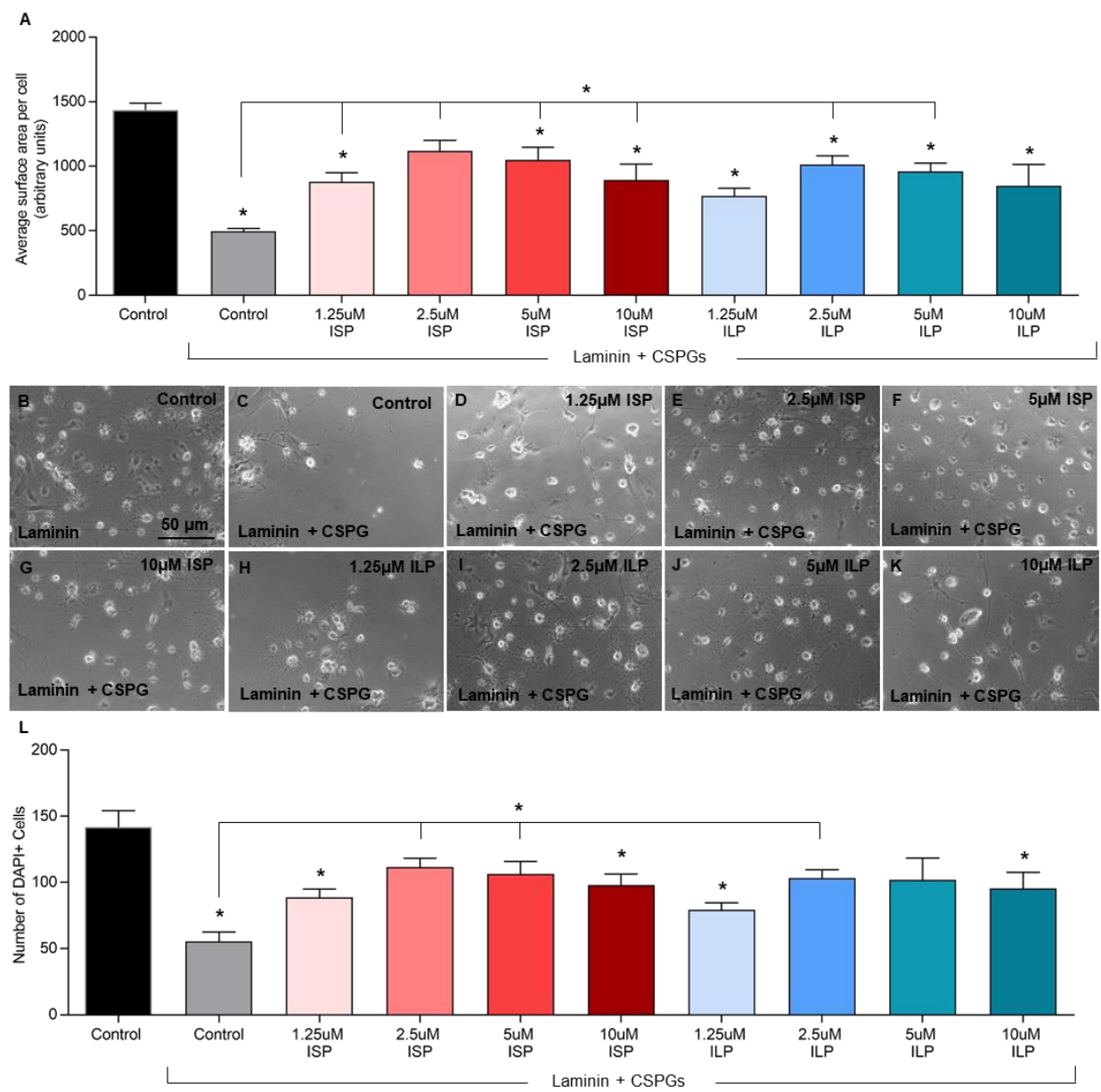
Supplementary Figure 3.2: ILP and ISP limit CSPG effects on OPCs attachment and cell spreading in a dose dependant manner.

OPCs were grown onto laminin (5μg/ml) or laminin (5μg/ml) + CSPGs (10μg/ml) substrate to mimic the normal and post-SCI matrix composition, respectively. (A-K) Bright field images of OPCs at 1 day after cell plating demonstrated limited attachment and cell spreading on CSPGs substrate (C) compared to laminin only (B). Inhibition of PTPσ (D-G) and LAR (H-K) with ISP and ILP, respectively, blocked CSPG effects on cell spreading in a concentration dependent manner. OPCs showed the greatest improvement in cell spreading when treated with 2.5μM ISP (D) and 2.5μM ILP (I). (L) Similarly, OPCs attachment was significantly decreased on CSPGs substrate that was partially stored with both ISP and ILP with the greatest effect seen by 2.5μM ISP (D) and 2.5μM ILP (I). N = 3 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

Supplementary Figure 3.3: ILP and ISP attenuate dephosphorylation of Erk1/2 and Akt signalling on CSPGs substrate in an additive manner.
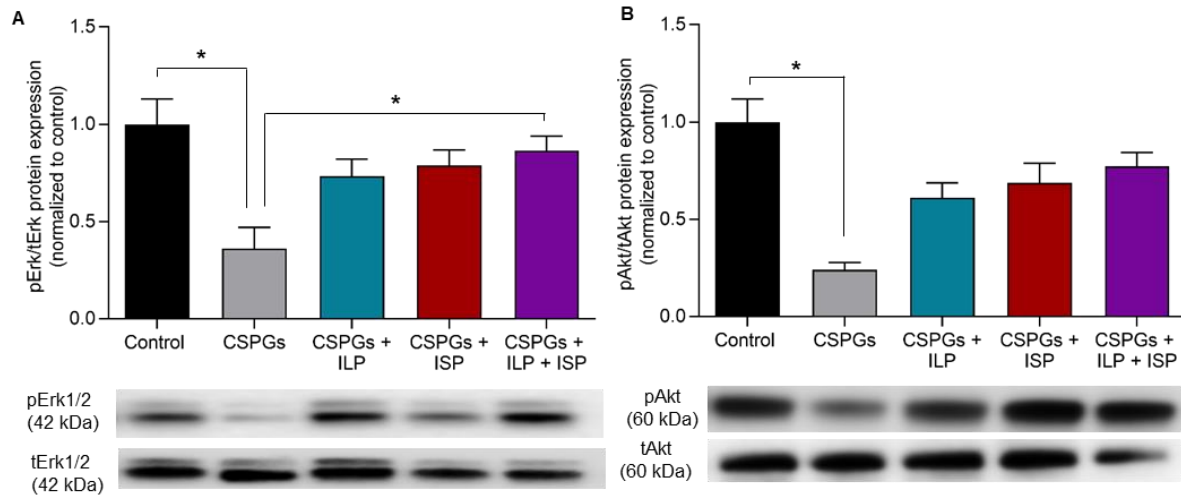
Spinal cord NPCs were grown on laminin (5μg/ml) or laminin (5μg/ml) + CSPGs (5μg/ml) substrate. Western blotting analysis of Erk1/2 (A) and Akt (B) phosphorylation was performed demonstrating that CSPGs significantly decrease Erk1/2 and Akt phosphorylation. This was partially overcome by ILP, ISP and ILP/ISP treatment 1 day following cell plating. However, only combination of ILP/ISP resulted in significant activation of pErk1/2. N = 3 independent experiments. The data show the mean ± SEM. * $P<0.05$, One Way ANOVA.

Supplementary Figure 3.4: Long-term functional stability of ILP and ISP at 37ºC.
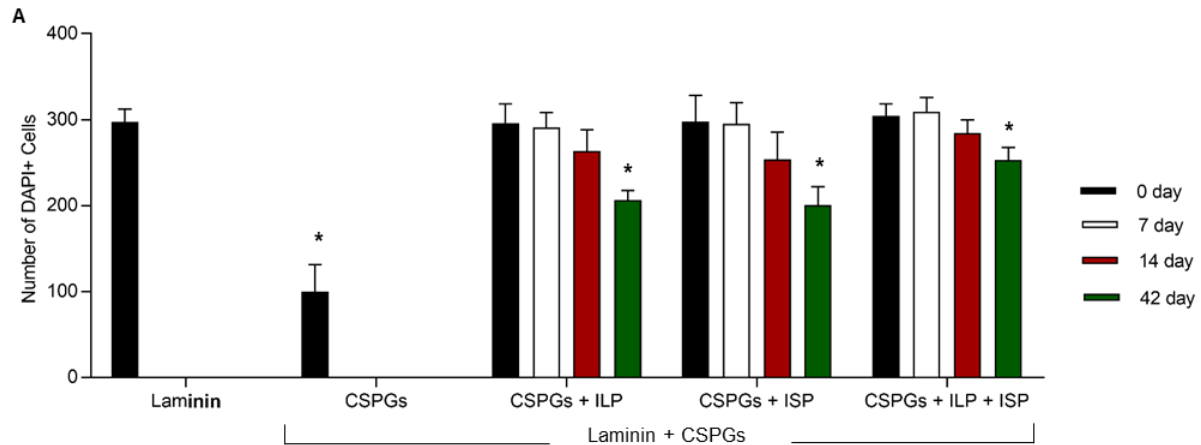
(A) We assessed the functional stability of ILP and/or ISP stored at 37ºC for a longer period of time. Both peptides were diluted and stored at 37ºC in the same manner as our *in vivo* delivery system in which ILP and ISP are packaged into osmotic pumps. We used our attachment assay on NPCs to assess the functionality of ISP and ILP. NPCs were grown on laminin (5µg/ml) or laminin (5µg/ml) + CSPGs (5µg/ml) substrate. Fresh or ILP/ISP stored for 7, 14 and 42 days at 37^{0}C was added to NPCs. As anticipated, CSPGs limited the ability of NPCs to attach as indicated by a decrease in the number of DAPI+ cells. ILP and ISP incubated at 37ºC was able to overcome CSPGs inhibitory effects at all time-points including 42 days. This indicates that ILP and ISP had preserved their bioactivity. N = 4 independent experiments. The data show the mean ± SEM. * P<0.05, One Way ANOVA.

Chapter 4: Perturbing Chondroitin Sulfate Proteoglycan Signaling through LAR and PTPσ Receptors Promotes a Beneficial Inflammatory Response following Spinal Cord Injury

This collaborative work has been published in the Journal of Neuroinflammation.

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Author contribution: I contributed to over 80% of the total experimental procedures, data and analysis and manuscript preparation. Intracellular Sigma Peptide (ISP) which was used in this study was created by Dr. Bradley Lang and Dr. Jerry Silver from Case Western University. Cell culture of Neural Stem/Precursor Cells was done by Santhosh Thomas Kallivalappil and microglia cell cultures were done by Hardeep Kataria. Flow cytometry experimental procedures were performed by Hardeep Kataria and myself and data analysis was performed by Arsalan Alizadeh.

Competing financial interest: SD, HK, SKT and SK-A declare no competing financial interests. BL and JS are inventors on the patent application (PCT/US2013/035831) for ISP.

4.1 Abstract

Background: Traumatic spinal cord injury (SCI) results in upregulation of chondroitin sulfate proteoglycans (CSPGs) by reactive glia that impedes repair and regeneration in the spinal cord. Degradation of CSPGs is known to be beneficial in promoting endogenous repair mechanisms including axonal sprouting/regeneration, oligodendrocyte replacement, and remyelination, and is associated with improvements in functional outcomes after SCI. Recent evidence suggests that CSPGs may regulate secondary injury mechanisms by modulating neuroinflammation after SCI. To date, the role of CSPGs in SCI neuroinflammation remain largely unexplored. The recent discovery of CSPG-specific receptors, leukocyte common antigen-related (LAR) and protein tyrosine phosphatase-sigma (PTPσ) allows unraveling the cellular and molecular mechanisms of CSPGs in SCI. In the present study, we have employed parallel *in vivo* and *in vitro* approaches to dissect the role of CSPGs and their receptors LAR and PTPσ in modulating the inflammatory processes in the acute and subacute phases of SCI.

Methods: In a clinically-relevant model of compressive SCI in female Sprague Dawley rats, we targeted LAR and PTPσ by two intracellular functionally blocking peptides, termed ILP and ISP, respectively. We delivered ILP and ISP treatment intrathecally to the injured spinal cord in a sustainable manner by osmotic mini-pumps for various time-points post-SCI. We employed flow cytometry, Western Blotting and immunohistochemistry in rat SCI, as well as complementary *in vitro* studies in primary microglia cultures to address our questions.

Results: We provide novel evidence that signifies a key immunomodulatory role for LAR and PTPσ receptors in SCI. We show that blocking LAR and PTPσ reduces the population of classically activated M1 microglia/macrophages, while promoting alternatively activated M2 microglia/macrophages and T regulatory cells. This shift was associated with a remarkable

elevation in pro-regenerative immune mediators, interleukin-10 (IL-10) and Arginase-1. Our parallel *in vitro* studies in microglia identified that while CSPGs do not induce an M1 phenotype *per se*, they promote a pro-inflammatory phenotype. Interestingly, inhibiting LAR and PTPσ in M1 and M2 microglia positively modulates their inflammatory response in the presence of CSPGs, and harnesses their ability for phagocytosis and mobilization. Interestingly, our findings indicate that CSPGs regulate microglia, at least in part, through the activation of the Rho/ROCK pathway downstream of LAR and PTPσ.

Conclusions: We have unveiled a novel role for LAR and PTPσ in regulating neuroinflammation in traumatic SCI. Our findings provide new insights into the mechanisms by which manipulation of CSPGs signaling can promote recovery from SCI. More importantly, this work introduces the potential of ILP/ISP as a viable strategy for modulating the immune response following SCI and other neuroinflammatory conditions of the central nervous system.

4.2 Background

Spinal cord injury (SCI) results in profound inhibitory modifications in the extracellular matrix (ECM), mainly driven by activated glia (Dyck and Karimi-Abdolrezaee 2015). Dysregulation of the ECM contributes considerably to the formation of an impermissible microenvironment for repair and regeneration after SCI (Cregg et al. 2014). Dramatic upregulation of chondroitin sulfate proteoglycans (CSPGs) is considered a main inhibitory component of the post-SCI ECM (Dyck and Karimi-Abdolrezaee 2015). CSPGs limit several endogenous repair mechanisms including axonal sprouting and regeneration as well as oligodendrocyte replacement and remyelination (Dyck et al. 2015; Bradbury et al. 2002; Karimi-Abdolrezaee et al. 2012; Karimi-Abdolrezaee et al. 2010; Barritt et al. 2006; Cafferty et al. 2008). Different strategies have been employed to target CSPGs after SCI such as administration of chondroitinase ABC (ChABC) and xyloside treatments and genetic manipulations of N-acetylgalactosaminyl-transferase 1 and Sox9 (McKillop et al. 2013; McKillop et al. 2016; Bradbury et al. 2002; Karimi-Abdolrezaee et al. 2010; Massey et al. 2006; Takeuchi et al. 2013; Rolls et al. 2008). Importantly, these studies have shown that inhibition of CSPGs improves recovery from SCI.

While the significance of CSPGs on spinal cord regeneration has been established, the cellular and molecular mechanisms of CSPGs in neuroinflammatory processes have yet to be elucidated. The recent discovery of specific CSPGs signaling receptors, leukocyte common antigen-related (LAR) and protein tyrosine phosphatase-sigma (PTPσ) (Shen et al. 2009; Fisher et al. 2011), provides the opportunity to uncover immunomodulatory mechanisms of CSPGs.

SCI triggers a complex immune response which is characterized by activation of resident microglia and recruitment of peripheral leucocytes to the site of injury. Currently, it is well accepted that neuroinflammation can be both beneficial and detrimental for SCI repair depending

on the timing and phenotype of immune cells following injury (Rust and Kaiser 2017; David and Kroner 2011; Miron and Franklin 2014). Classically activated M1 microglia/macrophages are known to promote tissue damage through their production of pro-inflammatory cytokines (i.e. of IL-1β, TNFα, IL-6, IL-12, IL-23, and IFN-γ), proteases, and reactive oxygen species (ROS) (Miron and Franklin 2014). Conversely, alternatively activated M2 microglia/macrophages are associated with phagocytosis of myelin debris and secretion of growth promoting factors (i.e. IL-10, IGF-1) that support tissue repair (Song et al. 2017; Ma et al. 2015; Miron and Franklin 2014). In mice SCI, there is initially a relatively equal number of M1 and M2 microglia/macrophage, which over the time shifts to an increasingly more prominent M1 inflammatory response (Kigerl et al. 2009). This switch is deleterious to endogenous repair mechanisms as an M2 immune response has been shown to be essential for multiple repair processes including axonal sprouting/regeneration in SCI (Song et al. 2017; Ma et al. 2015; Kitayama et al. 2011; Bollaerts et al. 2017) and oligodendrocyte maturation and remyelination in multiple sclerosis (MS) (Miron et al. 2013). The microenvironment of SCI appears to favour an M1 phenotype as transplantation of M2 macrophages into the injured spinal cord at 7 days post-SCI drives the majority of these cells to adopt an M1 phenotype 3 days post-transplantation (Rapalino et al. 1998; Kigerl et al. 2009; Kroner et al. 2014). Thus, a better understanding of the endogenous mechanisms that regulate immune cells in the injured spinal cord will allow for the development of immunomodulatory therapies for SCI.

Evidence suggests that targeting the upregulated levels of CSPGs by ChABC treatment can promote an M2 inflammatory response after SCI (Didangelos et al. 2014; Bartus et al. 2014). Here, we investigated whether CSPGs modulate inflammatory processes after SCI through the activation of LAR and PTPσ. Studies by our group and others have identified a critical role for LAR and

PTPσ in mediating CSPG effects on multiple cell types including neural precursor cells (NPCs) (Dyck et al. 2015), oligodendrocyte precursor cells (OPCs) (Pendleton et al. 2013) and neurons (Lang et al. 2015; Ohtake et al. 2016; Fisher et al. 2011; Shen et al. 2009), and genetic manipulation of their expression is sufficient to limit CSPG effects in vitro. Membrane permeable Intracellular LAR Peptide (ILP) and Intracellular Sigma Peptide (ISP) have been developed to inhibit LAR and PTPσ receptors (Lang et al. 2015; Xie et al. 2006). These peptides are designed to binds to a highly conserved 24-amino acid intracellular wedge domain and thereby block the catalytic activity of these receptors. Efficacy of ILP and ISP in blocking CSPG effects has been demonstrated in vitro and in SCI (Lang et al. 2015; Xie et al. 2006; Fisher et al. 2011). In rat SCI, our group and others have demonstrated that pharmacological inhibition of LAR and PTPσ by ISP and ILP remarkably increases sprouting of serotonergic fibers associated with improved functional recovery (Fisher et al. 2011; Lang et al. 2015).

In the present study, by utilizing ILP and ISP in a clinically-relevant model of compressive/contusive SCI in the rat, we have unveiled a novel immunomodulatory role for CSPGs that is mediated through LAR and PTPσ receptors. We show that blocking LAR and PTPσ with ILP and ISP fosters an increase in the number of M2 microglia/macrophages and T regulatory cells after SCI that is marked by elevated levels of interleukin-10 (IL-10). Our parallel in vitro studies on microglia uncovered that while CSPGs themselves do not induce an M1 phenotype, their presence in the milieu of M1 microglia promotes their pro-inflammatory phenotype. Importantly, inhibiting LAR and PTPσ in M1 microglia attenuated their IL-1β expression in the presence of CSPGs and promoted their phagocytic ability and mobilization. Moreover, we have identified that CSPGs regulate microglia, at least partially, by activation of the Rho/ROCK pathway in which can be attenuated by the inhibition of LAR and PTPσ. Taken together, our

findings provide novel insights into the cellular and molecular mechanisms by which modulation of CSPGs or their receptors, LAR and PTPσ, can improve endogenous repair mechanisms and neurological recovery following SCI (Lang et al. 2015; Fisher et al. 2011; Karimi-Abdolrezaee et al. 2010; Gaviria et al. 2002; Bradbury et al. 2002; Karimi-Abdolrezaee et al. 2012). We also provide the first evidence suggesting the potential of ILP and ISP as a candidate immunotherapy for SCI.

4.3 Materials and Methods

4.3.1 Animals and Animal care

All experimental protocols in this study were approved by the Animals Care Committee of the University of Manitoba in accordance with the guidelines and policies established by the Canadian Council of Animal Care (CCAC). For *in vivo* studies, a total of 112 adult female Sprague Dawley (SD) rats (250g), and for *in vitro* experiments, 6 C75BL/6 mice (8 weeks) and 44 postnatal (P1-P3) SD pups were used (provided by the Central Animal Care Facility at the University of Manitoba, Canada). Adult female rats were housed in standard plastic cages at 22°C before SCI and at 26°C afterwards in a 12:12 h light/dark cycle. Pelleted food and drinking water were available *ad libitum*. Hardwood sawdust bedding was used before SCI surgeries and replaced by

4.3.2 Model of Compressive Spinal Cord Injury

We employed a clinically relevant clip-compression model of SCI that has been extensively characterized and employed for SCI pathophysiology and therapeutics by our group and others (Rivlin and Tator 1977; Karimi-Abdolrezaee, Eftekharpour, and Fehlings 2004; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Gauthier et al. 2013). Under sterile

conditions, general anesthesia was induced by inhalation of a mixture of O_2 (2 liters per min) and Isoflurane (4%) via a mask integrated into a surgical stereotaxic frame. After deep anesthesia was achieved, for maintenance, isoflurane was reduced to 2%. The surgical area was shaved and disinfected with 70% ethanol and Povidone Iodine. A midline incision was made at the thoracic area (T4–T9) and skin and superficial muscles were retracted. The rats received a T6–T8 laminectomy and then, a 35g aneurysm clip (University Health Network, Toronto, Ontario, Canada) was applied for 1 min at the level of T7 of the spinal cord extradurally inducing a compression injury. For surgical pain and discomfort management, each animal received one single injection of meloxicam (Metacam® Boehringer Ingelheim GmbH, 2mg/kg) prior to the surgery followed by four doses of buprenorphine (Vetergesic, 0.03mg/kg) immediately after SCI with 8 hour (hr) intervals. Animals also received5 ml of 0.9% saline subcutaneously after SCI and thereafter as needed to prevent dehydration. Additionally, animals received oral Clavamox® (Amoxicillin plus Clavulanic Acid, Pfizer) in their drinking water starting two days before surgeries until 3 days post-operation to prevent trauma-induced hematuria and bladder infection. SCI rats were examined daily to monitor their recovery and their bladder was expressed manually three times a day until the return of reflexive bladder control.

4.3.3 Experimental Groups and Treatments

Prior to the SCI procedure, animals were randomly assigned to three experimental groups: 1) Uninjured; 2) SCI/vehicle control, receiving vehicle solution used for preparation of ILP + ISP (0.1% bovine serum albumin, BSA in 0.9% saline); 3) SCI/ ILP (NH2-GRKKRRQRRRCDLADNIERLKANDGLKFSQEYESI-NH2, CS Bio Co.) + ISP (NH2-GRKKRRQRRRCDMAEHMERLKANDSLKLSQEYESI-NH2, CS Bio Co.) (10μg each/day)

(Table 4.1). The dose of ILP and ISP was previously determined in our previous SCI studies (Lang et al. 2015). We conducted time-point analyses at 1, 3, 5, 7 and 14 days. For all time-points (Table 4.1), treatment was administered intrathecally at the time of SCI using a mini-osmotic pump and an indwelling intrathecal catheter inserted into the subarachnoid space surrounding the lesion site with the tip of the catheter located in the rostral region to the lesion (Alzet –model 2001D, 1003D, 2001, 2002 and 2004). Based on the delivery rate of each pump, ILP and ISP concentration was adjusted to ensure sustained delivery of comparable dose per day for each time-point. SCI/vehicle control group also received vehicle via osmotic pumps in the same manner as the treatment groups. Uninjured animals did not undergo any surgical procedure in this study.

Table 4.1. Summary of experimental procedures, treatment groups, and time-points for *in vivo* experiments.

SCI + treatment regimen starting at the time of injury	Time-points for tissue analysis					
Day 0	1 day	3 days	5 days	7 days	14 days	28 days

Treatment given for entire study until endpoint

Western Blot: 1, 3, 5, 7 and 14 days post injury
G-LISA: 3 days post injury
Flow Cytometry: 3 and 7 days post injury
Immunohistochemistry: 28 days post injury

Experimental Groups:
1) Uninjured Control
2) SCI + Vehicle (saline + BSA)
3) SCI + ILP (10μg/day) + ISP (10μg/day)
Sample size:
N= 4-6 rats/group/time-point

4.3.4 Tissue Processing

Tissue harvesting was performed at the end of treatment period. Animal that received treatment at the time-point of injury were euthanized at 1, 3, 5, 7, 14, and 28 days post-SCI (N = 4-6 animals/group/time-point). SCI rats were deeply anesthetized with a mixture of 40% isoflurane/60% propylene glycol (Fisher Scientific, Pittsburgh, PA, USA). For molecular analyses, rats were perfused transcardially with 0.1M phosphate buffered saline (PBS) to remove blood. Once blood was removed, freshly dissected spinal cords were placed in ice-cold aCSF, and cleaned of meninges and nerve roots. Five millimeters of the spinal cord tissue centered at the injury site was dissected and processed either for Western blotting or slot blotting, as described in subsequent sections. For histological analyses, under deep anesthesia, rats were perfused transcardially with 2.5% paraformaldehyde (PFA) in 0.1M PBS, pH 7.4. The spinal cords were excised and subsequently postfixed in the perfusing solution plus 10% sucrose overnight at 4°C. Then, the tissues were further cryoprotected in 20% sucrose in PBS for 24-48 hours at 4°C. A 1.5-2 cm length of the spinal cord tissue centered at the injury site was dissected and embedded in tissue embedding medium (Tissue-Tek™ CRYO-O.C.T Compound, Electron Microscopy Sciences) on dry ice. Cross sections (35µm) were cut serially on a cryostat (Leica) and mounted onto Superfrost® Plus Micro Slides (Fisher Scientific) and stored at -80°C until immunostaining procedure.

4.3.5 Immunohistochemistry on Tissue Sections

Frozen spinal cord cross-sections were air-dried at room temperature for 30 minutes. The injury epicenter for each sample was determined by Hematoxylin and Eosin (H/E) staining and the section near the midpoint of the lesion with the largest injury area was considered as the epicenter.

Slides were permeabilized with PBS for 5 minutes and then blocked for 1 hr at room temperature using 5% skim milk, 1% BSA, 0.05% Triton-X in 0.1M PBS. This blocking solution was used for all immunostaining procedures unless otherwise has been mentioned. Tissue sections were then incubated overnight at 4°C with primary antibodies (Table 4.2) diluted in the blocking solution. Sections were washed three times in PBS then incubated with Alexa 568 goat anti-mouse, rabbit, or goat secondary antibody (1:400; Invitrogen) for 1.5 hr. In double staining procedure, the tissue sections were treated with a second primary antibody then incubated with Alexa 488 goat anti-mouse or rabbit secondary antibody (1:400; Invitrogen). The slides were washed three times with PBS and incubated with DAPI as a nuclear counterstain.

Table 4.2. List of antibodies used in this study

Note: ICC: immunocytochemistry, IHC: immunohistochemistry, WB: Western blot

Antibody	Source	Usage	Dilution factor
Actin	Sigma (Rabbit, A2066)	WB	1:300
Actin	Chemicon (Mouse, MAB1501R)	WB	1:300
Arginase-1 (5% BSA Blocking)	Cell Signaling (Rabbit, 9819S)	WB	1:1000
BrdU	Santa Cruz (Mouse, 555627)	ICC	1:500
CD3 (1% BSA, 5% goat serum blocking)	Abcam (Rabbit, AB5690)	IHC	1:200
CD4	Cedarlane (Sheep, AF6439)	WB	1:100
CD86	Abcam (Rabbit, AB53004)	ICC	1:60
CS56	Sigma (Mouse, C8035)	IHC	1:150
DAPI	Sigma (D9542)	ICC IHC	1:10000
FOXP3	Cedarlane (Rat, 14-5773-82)	WB IHC	1:200 1:100
Iba1	Wako (Rabbit, 016-20001)	WB	1:1000
Iba1	Wako (Rabbit, 019-19741)	ICC IHC	1:500 1:500
IL-10	Cedarlane (Mouse, MAB519)	WB	1:250
IL-10 (1% BSA, 5% Goat serum blocking)	R&D Systems (Rat, MAB417)	IHC	1:200
IL-1β	Serotec (Rabbit, AAR15G)	WB	1:1000
GFAP	Chemicon (Mouse, MAB360)	ICC	1:800
GFAP	Cell Signaling (Mouse, #3670S)	WB	1:5000
GFAP	Chicken	IHC	1:800
GAPDH	Santa Cruz (Rabbit, sc-25778)	WB	1:1000
LAR	Santa Cruz (Rabbit, sc-25434)	ICC	1:50
LAR	BD (Mouse 610351)	WB	1:250
Mannose Receptor	Abcam (Rabbit, ab64693)	ICC	1:400
Olig2	Chemicon (Rabbit, AB9610)	ICC	1:2000
OX42 (CD11b)	Serotec (Mouse, MCA275G)	IHC	1:100
PTPσ	R&D (Goat, AF3430)	ICC	1:50
TNFα	Serotec (Rabbit, AAR33)	WB	1:1000

4.3.6 Assessment of GFAP and CS56 Immunointensity in SCI

CSPGs and glial fibrillary acidic protein (GFAP) immunostaining was imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) (n = 4-6 animals/group/time-point). We imaged the entire cross-section of the spinal cord with a 10x objective using Zen tiling software (Zeiss). Imaging procedures were conducted under the same condition and consistent exposure time for all spinal cord sections as we described previously (Gauthier et al. 2013; Karimi-Abdolrezaee et al. 2010; Alizadeh 2017). Using NIH ImageJ software (imagej.nih.gov), we traced the cross-sectional area of the spinal cord and measured the immunodensity of GFAP and CSPGs in spinal cord cross-

sections representing the injury epicenter as well as 1mm rostral and caudal to the lesion center. After setting the threshold automatically, immunodensity above threshold was calculated. To account for variation in the size of spinal cord cross sections, the following formula was used to calculate the percentage of CSPG and GFAP area: normalized immunodensity of tissue section X = total immunodensity of tissue section X / total area of the spinal cord section X.

4.3.7 Western Blotting

For Western blotting, spinal cord tissue or cultured cells were homogenized in RIPA buffer (Thermo Fisher) containing SigmaFast Protease Inhibitor (Sigma). A total of 10µg to 50µg of proteins were then loaded onto a gel and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were then blocked in 5% non-fat milk in Tween Tris Buffered Saline (TTBS) and incubated overnight at 4°C with different antibodies (Table 4.2) diluted in the blocking solution. The membranes were washed and incubated with HRP-conjugated goat anti-mouse, anti-sheep, anti-rat or anti-rabbit antibodies (1:4000, Bio-Rad). Membranes were then incubated in ECL plus immunoblotting detection reagents (Thermo Scientific) according to the manufacturer's specifications. For arginase-1 antibody, the blocking solution was made of 3% BSA in TTBS. Immunoreactive bands were quantified using AlphaEaseFC (FluorChem, 8900). To control for equal protein loading, membranes were re-probed for actin antibody.

4.3.8 Myeloperoxidase Assay in SCI

Myeloperoxidase (MPO) enzyme activity was assessed as previously described (Suzuki et al. 1983). Briefly, MPO assay buffer was prepared by dissolving 80mmol phosphate buffer (pH: 5.4), 0.5% hexadecyltrimethyl ammonium bromide, and 1.6mmol tetramethylbenzidine in

dimethylformamide and 2mmol H_2O_2. Then, 200µl of MPO assay buffer was added to 50µg of tissue lysate at 37°C. Change in absorbance per minute was assessed at 655nm. MPO activity was expressed as the amount of the enzyme producing one absorbance change per minute.

4.3.9 Gelatin Zymography on SCI tissues

To assess matrix metalloproteinases (MMP) -2 and MMP-9 enzymatic activity in the injured spinal cord tissue, 50µg of protein obtained from the SCI tissue was loaded on 10% SDS-polyacrylamide gel, copolymerized with 1 mg/ml gelatin as substrate and were separated by electrophoresis. Proteins were renatured by 2.5% Triton X-100 to restore gelatinase activity. Gels were then incubated with developing buffer for 48-72 hours at 37°C to allow gelatinase activity of MMP-2 and MMP-9. Gels were stained with Coomassie blue for 30 minutes and de-stained in 30% Ethanol/10% acetic acid until appropriate color contrast was achieved. Areas of gelatinase activity appeared as clear bands against a dark blue background. MMPs were identified based on their molecular weight and their density was measured as described in our Western blot procedures.

4.3.10 Flow Cytometric Assessment in rat SCI

To study changes in immune cell population and phenotypes in the injured spinal cord, we performed flow cytometry on spinal cord tissue (Nguyen, Beck, and Anderson 2011). At 3 and 7 days post-injury, (N=5/experimental group), animals were anesthetized and euthanized. The vertebral columns were excised and placed on dry ice for 5 minutes. The spinal cords were exposed using a laminectomy and 1.5cm of tissue centered at the injury epicenter was excised, minced and enzymatically dissociated by incubating with 2.5mg trypsin + 5mg collagenase in 5ml DMEM media for 20min at 37°C. Cells were pelleted and reconstituted in 6ml of HBSS and overlaid on

OptiPrep® (Sigma-Aldrich, D1556) gradient for separation of myelin debris and incubated with red blood cell (RBC) lysis buffer (Biolegend, 420301) prior to counting. An average of 7.5 million cells was harvested from each animal. For each antibody panel, 2 million cells per animal were used. Non-specific binding sites were blocked using 10% normal mouse serum for 30min (Invitrogen, 10410). Cells were then incubated in an antibody cocktail containing surface antibodies for each panel for 30min away from light at 4°C. Next, cells were washed and fixed using BD^{TM} Cytofix Fixation Buffer for 15min at 4°C (BD, 554655). To stain for intracellular markers, cells were incubated with permeabilizing buffer (0.1% saponin + 10% FBS in HBSS) for 30min then incubated with a cocktail of intracellular antibodies for 30min in the dark. After washing, cells were reconstituted with 500µl of flow cytometry staining buffer and analysed using BD FACS Canto II flow cytometer counting 200,000 events per sample. Compensation was done prior to acquisition using single stained beads (OneComp eBeads, 501129031, eBioscience). For each antibody panel, proper isotype controls were used to account for non-specific antibody binding (Supplementary Fig. 4.1-4.2). Our flow gating strategy is depicted in Supplementary Figures 4.3-4.4. Additionally, flow cytometry antibodies and their isotype controls are listed in Table 4.3.

Table 4.3. List of flow cytometry antibodies used in this study.

Antibody	Color	Company-Cat number	Dilution Factor
CD3	PerCP	eBioscience, 46-00-30-82	1:20
CD4	BV510	BD, 740138	1:20
Ms IgG2a, k	BV510	BD, 563027	1:20
CD45	APC-Cy7	BD, 561586	1:20
Ms IgG1, k	APC-Cy7	BD, 557873	1:20
IFN-γ	FITC	BD, 559498	1:20
Ms IgG1, k	FITC	BD, 554679	1:20
IL-10	PE	BD, 555088	1:20
Ms IgG2b, k	PE	BD, 555058	1:20
FoxP3	APC	eBioscience, 17-5773-80	1:20
Ms IgG2a, k	APC	eBioscience, 17-4724-42	1:20
CD68	FITC	Bio-Rad, MCA341F	1:20
Ms IgG1	FITC	Bio-Rad, MCA1209F	1:20
CD163	PE	Bio-Rad, MCA342R	1:20
Ms IgG1	PE	Bio-Rad, MCA1209PE	1:20
CD86	BV-421	BD, 743211	1:20
Ms IgG1, k	BV-421	BD, 562438	1:20
IL-10	Alexa 647	BD, 562156	1:20
Ms IgG2b, k	Alexa 647	BD, 557903	1:20
TNFα	PE-Cy7	eBioscience, 25-7423	1:20
Armenian Hamster IgG isotype control	PE-Cy7	eBioscience, 25-4888-82	1:20

4.3.11 Culture and Isolation of Primary Microglia

Primary Microglia were isolated from mixed glial cultures as described previously (Saura, Tusell, and Serratosa 2003). Briefly, postnatal (P1-P3) rat pup cortices were mechanically dissociated and the cells passed first through a 70μm and then a 45μm cell strainer. The cell solution was then plated into 75 cm^2 flasks in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum and 1% PSN. Mixed glia cultures were maintained with media change every three days until confluency (2-3 weeks). Mixed glia cultures were then shaken at 200 rpm for 6 hr at 37°C to separate microglia from underlying astrocytes. The microglia were seeded over poly-D-Lysine (PDL, 0.1mg/ml, Sigma) coated dishes in 50% fresh media (DMEM/F12 plus 10% FBS) and 50% mixed glia conditioned media (collected and filter sterilized after shaking). Purity of microglia in these cultures was over 90% (data not shown).

4.3.12 Microglia Polarization

Primary microglia were plated at a density of 500,000 cells per well of a 6 well plate in 50% glia conditioned media and 50% DMEM/F12 plus 10% FBS media. Once the majority of microglia were attached and began to spread their cell processes (after 1-2 days), their media was changed and microglia were polarized to M0 (untreated), M1 (co-treatment of tumor necrosis factor-α (TNF-α, 40ng/ml) and interferon gamma (IFNγ, 50ng/ml), and M2 (interleukin-10 (IL-10, 10ng/ml). Two days following cell activation microglia conditioned media was harvested and stored at -80 for future experiments. Microglia polarization was confirmed using ELISA, the Griess assay and immunocytochemistry analyses for various M1 and M2 markers.

4.3.13 Plating Microglia on CSPGs Substrate

All tissue culture dishes were first coated with PDL (0.1mg/ml, Sigma) overnight at room temperature, followed by CSPGs (5µg/mL, Millipore, cc117) for 6 hours at 37°C as we described previously (Dyck et al. 2015). Of note, a mixture of CSPGs used in this study contained a mixture of neurocan, phosphacan, versican, and aggrecan. Where appropriate, chondroitinase ABC (ChABC, 0.1 U/ml Sigma, C3667-10UN) was added to the CSPGs mixture during the coating step. M0, M1, and M2 microglia cultures were activated three hours prior to cell plating (see Table 4.4). Three hours following cell activation, microglia were lifted and plated onto various substrates including: 1) PDL; 2) PDL + CSPGs (5µg/ml); 3) PDL + CSPGs (5µg/ml) + ChABC. Two days following treatments microglia were assessed for various outcomes including cytokine release, nitric oxide activity, and phagocytosis.

Table 4.4. Assessment of CSPG effects on Microglia Experimental Groups

Experimental Group	Treatment	Cell Plating (3 hours post treatment)
M0 (no treatment)	-	1) PDL; 2) PDL + CSPGs (5µg/ml) 3) PDL + CSPGs (5µg/ml) + ChABC (0.1U/ml)
	ILP (10µM) + ISP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	Y-27632 (ROCK Inhibitor, 10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	TAT (20µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	IMP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
M1 (40ng/ml TNFα + 50ng/ml IFNγ)	-	1) PDL; 2) PDL + CSPGs (5µg/ml) 3) PDL + CSPGs (5µg/ml) + ChABC (0.1U/ml)
	ILP (10µM) + ISP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	Y-27632 (ROCK Inhibitor, 10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	TAT (20µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	IMP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
M2 (10ng/ml IL10)	-	1) PDL; 2) PDL + CSPGs (5µg/ml) 3) PDL + CSPGs (5µg/ml) + ChABC (0.1U/ml)
	ILP (10µM) + ISP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	Y-27632 (ROCK Inhibitor, 10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	TAT (20µM)	1) PDL 2) PDL + CSPGs (5µg/ml)
	IMP (10µM)	1) PDL 2) PDL + CSPGs (5µg/ml)

4.3.14 *In vitro* Assessment of ILP and ISP Peptides in Blocking CSPG effects on Microglia

ILP and ISP peptides, against LAR and PTPσ, respectively, were used (10µM) as we described previously (Lang et al. 2015). A control peptide Intracellular Mu Peptide (IMP, NH2-LLQHITQMKCAEGYGFKEEYESGRKKRRQRRRC-NH2, CS Bio Co.) was also used to assess specificity of ILP and ISP effects (Xie et al. 2006). All peptides contained a transactivator of transcription of human immunodeficiency (TAT), domain (GRKKRRQRRRC) to facilitate intracellular delivery. Additionally, our previous work in NPCs revealed CSPGs signaling is

mediated through the Rho/ROCK pathway (Dyck et al. 2015). As a result, we also treated microglia with Y-27632 (10µM), a ROCK inhibitor in our microglial cultures. Microglia were dissociated and plated onto tissue culture surfaces containing one of the two conditions: 1) PDL; 2) PDL + CSPGs. For both experimental conditions, microglia were pretreated with IMP (control), TAT peptide (control), Y-27632, ILP, ISP, or ILP and ISP for 30 min.

4.3.15 Microglial Phagocytosis Assay

For the phagocytosis assay, green fluorescent latex beads of 1µm diameter (Sigma, L1030) were preopsinized by adding 1µl of the beads to 5µl of FBS for 1 hr at 37°C (Lian et al. 2016). Fluorescent beads were then diluted in microglia media and added to microglia cultures at a final concentration of 0.01% (v/v). After 1 hr, microglia media was removed and cells were washed twice gently with PBS then fixed in 3% PFA for 15 minutes. Microglia were stained with DAPI for 15 minutes and imaged. The percentage of DAPI+ cells containing fluorescent beads was quantified. Engulfment of fluorescent beads was confirmed by Z-stack imaging and co-localization of Iba1+ cells with green fluorescent signal.

4.3.16 Griess Nitrite Assay

The Griess assay (Promega, Fisher) was used to measure nitrite levels as a representative of NO activity in microglia conditioned media (MCM) collected at 48 hours after microglial activation according to the manufacturer's instructions (Green et al. 1982; Hu J 1996). To eliminate any possible interference in Griess assay readings, phenol red free media was used for these experiments.

4.3.17 Microglia Migration Assay

Microglia were plated onto PDL-coated or PDL + CSPGs (5µg/ml) poly-carbonate transwell culture inserts (Corning, 100,000 cells per transwell) in SFM in a 24 well plate. Microglia SFM containing C5a (R&D Systems, CL7336R 30nM) was added to the bottom chamber to act as a chemoattractant (Miller and Stella 2009). Cells were allowed to migrate for 16 hours at 37°C and were fixed for 30 minutes with 3% PFA and stained for DAPI (1:5000). Non-migrated cells on the upper side of the transwell were gently scraped off with a cotton swab and the migrated cells were visualized by DAPI staining as described previously (Miron et al. 2013). Eight images were taken at 40x magnification to determine the total number of DAPI+ migrated cells.

4.3.18 ELISA

We used commercial ELISA kits for cytokine analysis *in vitro* (DuoSet ELISA Development System; R&D Systems; #DY522 for IL-10; #DY501 for IL-1β) to specifically detect IL-10 and IL-1β in microglia conditioned media. The assay was performed according to the manufacturer's instructions, with standards (62.5-4000pg/ml for both assays) and loading of 50µl of microglia conditioned media per sample.

4.3.19 G-LISA

GTP bound RhoA was assessed with a G-LISA assay (Cytoskeleton, Inc. Denver, CO, USA). For *in vivo* tissue samples, spinal cord tissue was excised from the spinal cord at 3 days post injury and 1cm of spinal cord tissue centered at the injury epicenter was flash frozen in liquid nitrogen. For *in vitro* Rho activity assessment, TNFα (40ng/ml) + IFNγ (50ng/ml) treated M1 microglia were

grown on PDL or PDL + CSPGs with and without ILP (10μM) and ISP (10μM) for 1 day then harvested on ice and flash frozen in liquid nitrogen. For G-LISA assessment, 50μg of protein was used for both *in vivo* and *in vitro* assessments. G-LISA was performed as per manufacturer's instructions.

4.3.20 Isolation and Culturing of Spinal Cord Adult Neural Precursor Cells

Adult NPCs were isolated from the spinal cord of C57BL/6 mouse (8 weeks of age) as we described previously (Gauthier et al. 2013). Briefly, mice were deeply anesthetized by placing in a bell jar saturated with a mixture of 40% isoflurane/60% propylene glycol. Deep anesthesia was confirmed by lack of pedal reflexes. Mice were then killed by decapitation, and their spinal cords were excised under sterile conditions and transferred to artificial cerebrospinal fluid (aCSF) solution (containing 124mM NaCl, 3mM KCl, 1mM NaHPO4, 26mM NaHCO3, 1.5mM MgSO4, 1.5mM CaCl2, and 10 mM glucose). Spinal cords were cleaned of meninges and nerve roots, and were then subjected to a papain enzymatic solution (Worthington Biochemical Corporation) for 50 min at 37°C. The solution was next replaced by a papain inhibitor mixture and cells were passed through a 70µm cell strainer. Cellular components were isolated through 7.5% BSA gradient and resuspended in serum free medium (SFM, 100 ml) containing 84ml of DMEM/F12 (Invitrogen), 2ml of 30% glucose, 1.5ml of 7.5% NaHCO$_3$, 0.5ml of 1M HEPES, 10mg of transferrin, 2.5mg of insulin, 0.96mg of putrescine, 1µl of selenium, 1µl of progesterone, 1% L-glutamine, 1% penicillin/streptomycin/neomycin (PSN) and growth factors: 1µg of fibroblast growth factor-2 (FGF2, Fisher, CB40060A), 2µg of epidermal growth factor (EGF, Sigma, E-4127), and 200µg of Heparin (Sigma, H-3149). SFM plus growth factors will be referred as growth medium in the text.

Cells were plated onto uncoated tissue culture flasks (Biolite, Fisher Scientific). The neurospheres that were generated were passaged weekly by mechanical dissociation in growth medium.

4.3.21 Assessing the Effects of IL-10 on NPCs

NPC neurospheres were dissociated into single cells and plated onto PDL coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) in SFM. At 1 day following cell plating the media was changed and an IL-10 dosing assay was performed under the following conditions: 1) Control (SFM with no treatment); 2) 10ng/ml IL-10; 3) 50ng/ml IL-10; 4) 100ng/ml IL-10; 5) 200ng/ml IL-10 6) 400ng/ml IL-10. Assessment of NPC proliferation was performed in SFM for 1 day and NPC differentiation in 2% FBS for 7 days as we previously described (Gauthier et al. 2013; Dyck et al. 2015). For proliferation assessment, bromodeoxyuridine (BrdU 20µM, Sigma) was added to the cultures 3 hours before processing NPCs for immunocytochemistry. IL-10 neutralizing antibody was tested at various concentrations to determine the optimal concentration needed to block IL-10 effects on NPCs.

4.3.22 Assessing the Effects of Microglia Conditioned Media on NPCs

NPC neurospheres were dissociated into single cells and plated onto PDL coated multi-chamber glass slides (25,000 cells per chamber) (LabTek II) in SFM. One day following cell plating the media was changed to 50% NPC SFM and 50% MCM. The following experimental groups were assessed: 1) MCM alone- 50% NPC SFM + 50% fresh microglia media; 2) M0- 50% NPC SFM + 50% M0 MCM; 3) M1- 50% NPC SFM + 50% M1 MCM; 4) M2- 50% SFM + 50% M2 MCM. The polarizing factors used to convert microglia to an M1 and M2 phenotype were used as controls at their respective concentrations. IL-10 neutralizing antibody (R&D Systems, MAB417,

0.8µg/ml) was used to assess the overall effect of IL-10 on NPCs proliferation and differentiation. Assessment of NPC proliferation (1 day post MCM treatment) and differentiation (7 days post MCM treatment) was accomplished as described above.

4.3.23 *In vitro* Immunostaining, Image Processing and Analysis

For immunocytochemistry, cultures were fixed with 3% paraformaldehyde (PFA) for 20 minutes at room temperature and washed three times with PBS. Cells were incubated in a blocking solution containing 5% non-fat milk, 1% BSA, and 0.5% Triton X-100 in 0.1M PBS for 1 hr. Cultures then underwent an immunostaining procedure using methods described earlier for immunohistochemistry. For BrdU immunodetection, prior to blocking, sections were washed with PBS, incubated in 2N HCl and 0.5% Triton X-100 for 30 minutes at 37°C, and washed with 0.1M sodium borate in PBS for 10 minutes. After blocking, the slides were incubated with primary antibodies overnight and secondary antibodies were added as was previously described. For immunocytochemistry quantification, 8-10 separate fields (under 20X objective) containing an average of 300 cells for each condition were randomly imaged using a Zeiss AxioObserverZ1 inverted microscope or a Zeiss Imager 2 epi-fluorescence microscope. For each condition, the total number of DAPI positive cells was first assessed, and the number of positive cells for Olig2, GFAP, and BrdU (containing a DAPI positive nucleus) were then counted. The percentage of abundance of each cell type was calculated by dividing the number of positive cells for the marker by the total number of DAPI+ cells under each experimental condition. Values were then normalized to control condition for relative comparison.

4.3.24 Statistical Analysis

Using SigmaStat Software (4.0), we performed One Way ANOVA followed by Holm-Sidak post-hoc was used in all Western blot, MPO, MMP, and *in vitro* analyses. The student t-test was used when two groups were compared. The data was reported as means ± standard error of the mean (SEM). P≤0.05 was considered statistically significant.

4.4 Results

4.4.1 Inhibition of LAR and PTPσ Fosters a Beneficial Inflammatory Response by resident microglia and infiltrating leukocytes following SCI

Recent evidence suggests that degradation of CSPGs with ChABC promotes an anti-inflammatory M2 microglia/macrophage response after SCI (Bartus et al. 2014; Didangelos et al. 2014). Here, we sought to determine the role of LAR and PTPσ receptors in neuroinflammation in rat SCI using functionally blocking peptides against these receptors. We delivered TAT-conjugated ILP and ISP (10µg each/day) intrathecally to the injured spinal cord in a sustainable manner using an Alzet osmotic pump. The dose of ILP and ISP was previously determined by our group in a contusive model of rat SCI (Lang et al. 2015). ILP and ISP treatments were co-delivered in this study as our previous in vitro findings revealed that both receptors mediate CSPG effects and their combinatorial downregulation resulted in the greatest inhibition of CSPG effects in NPCs versus their solitary administration (Dyck et al. 2015). Infusion of ILP and ISP began immediately after SCI for a duration of 1, 3, 5, 7, or 14 days post-SCI (N = 4-6 animals/group/time-point). Stability of ILP and ISP in Alzet osmotic pumps at 37°C has previously been verified by our group confirming both ILP and ISP are stable for at least 42 days (data not shown). We performed a

battery of tissue assessments to study the impact of LAR and PTPσ receptors on immune response in rat SCI that are described in this section.

4.4.1.1 *Neutrophils*: Neutrophils are the first leukocytes infiltrating the SCI lesion peaking at 24 hours post-injury and remaining for up to 3 days (Taoka et al. 1997; Neirinckx et al. 2014). Neutrophil recruitment is associated with a pro-inflammatory phenotype that causes cell death and poor motor recovery (Kang et al. 2011; Neirinckx et al. 2014). We assessed neutrophils infiltration in SCI by measuring MPO activity, a well-established marker for assessment of neutrophils (Suzuki et al. 1983). While there was no detectable MPO activity in the baseline uninjured group, we found an elevated level of MPO at 1 day post-SCI that was significantly decreased under ILP/ISP treatment suggestive of reduced neutrophil infiltration (Fig. 4.1A, N=4-6 animals/group/time-point, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). At 3 days post-SCI, elevated MPO activity was generally decreased in the injured spinal cord to a level closer to the baseline uninjured group as expected, and there was no difference between vehicle and ILP/ISP treatment groups (Fig. 4.1A). Additionally, we studied the activity of MMPs in response to ILP/ISP treatment (N=4-6 animals/group/time-point). MMPs are upregulated and activated following SCI and contribute to blood-spinal-barrier degradation and influx of leukocytes into the injured tissue (Zhang et al. 2011). Using gelatin zymography, we detected a robust increase in MMP-9 at 1 and 3 days and in MMP-2 at 3 and 7 days post-SCI (Fig. 4.1B-D, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). However, ILP and ISP treatment had no apparent effect on modulating the activities of MMP-2 and MMP-9 after SCI in our model.

Figure 4.1: ILP/ISP treatment limits neutrophil infiltration but does not modulate MMP activity in acute SCI.
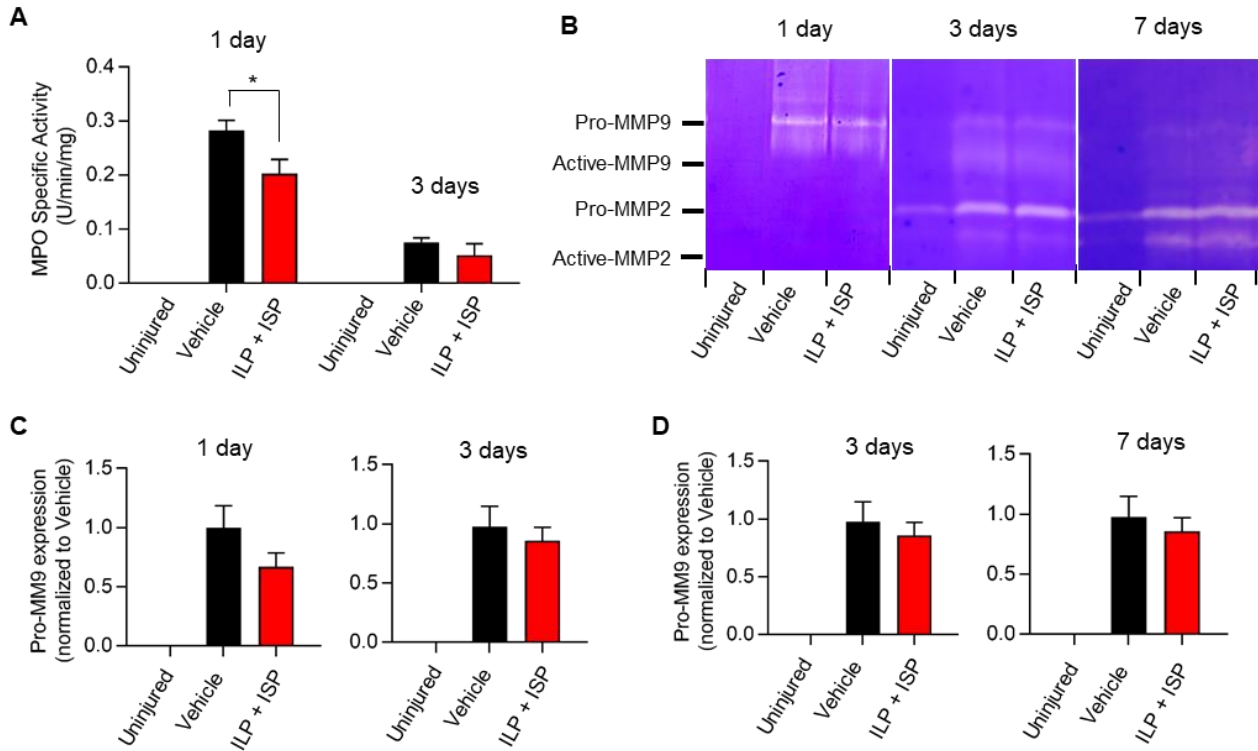
(A) Myeloperoxidase (MPO) activity, a marker for neutrophils, was increased in the injured spinal cord at 1 day following SCI, which was attenuated by ILP/ISP treatment. (B-D) Matrix metalloproteinases (MMP)-2 and MMP-9 expression was also assessed using gel zymography. SCI induced expression of MMP-2 and MMP-9 was observed at 1, 3 and 7 days post-SCI. However, there was no change in the levels of pro-MMP-9 (C) and pro-MMP-2 (D) under ILP/ISP treatment at any examined time-points. N = 4-6 animals/group/time-point. The data show mean ± SEM, *p<0.05, One Way ANOVA (A-D),

4.4.1.2 *Microglia/Macrophages:* Resident microglia and infiltrating blood born macrophages are key immune cells that increase their cell numbers in SCI peaking around 7 days post-injury (Popovich, Wei, and Stokes 1997; Hausmann 2003; Shechter et al. 2009). Our analysis of the microglia/macrophages marker Iba-1 at 1, 3, 5, 7 and 14 days post-SCI (N=4-6 animals/group/time-point) revealed that ILP/ISP treatment has no effect on the overall recruitment of microglia/macrophages to the SCI lesion (Fig. 4.2A, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Similarly, our flow cytometric analysis (N=5 animals/group/time-point) confirmed no apparent change in the overall infiltration of CD45+/CD68+ macrophages at both 3 and 7 days post-injury (Fig. 4.2B-C, $p < 0.05$, Student *t* test). Although the number of macrophages remained unaffected, ILP/ISP therapy was able to induce a phenotype shift from a predominantly M1 to a majority M2 profile. We observed a non-significant decrease in the number of CD45+/CD68+/CD86+ M1 macrophages at 3 and 7 days post-injury between vehicle and ILP/ISP treated animals (Fig. 4.2D-E, $p < 0.05$, Student *t* test). Conversely, ILP/ISP treated rats showed a significantly higher number of CD45+/CD68+/CD163+ M2 macrophages in their spinal cord at 7 days post-injury (Fig. 4.2F-G, $p < 0.05$, Student *t* test). Our cytokine analysis by Western blotting also reaffirmed our flow cytometry data and showed a reduction in pro-inflammatory cytokines, interleukin (IL)-1β and TNFα in ILP/ISP treated animals as compared to vehicle control group, which was significantly different at the 3-day time-point for IL-1β (Fig 4.3A-B, N=4-6 animals/group, $p < 0.05$, One Way ANOVA, Holm-Sidak *post hoc*). Instead, ILP/ISP treatment resulted in a marked increase in the production of IL-10 and Arginase-1, two well-known M2 associated markers that was statistically significant at 5, 7, and 14 days post-SCI compared to SCI vehicle treated group (Fig. 4.3C-D, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*).

Figure 4.2: Inhibition of LAR and PTPσ promotes an increase in the subpopulation of M2 macrophages after SCI.
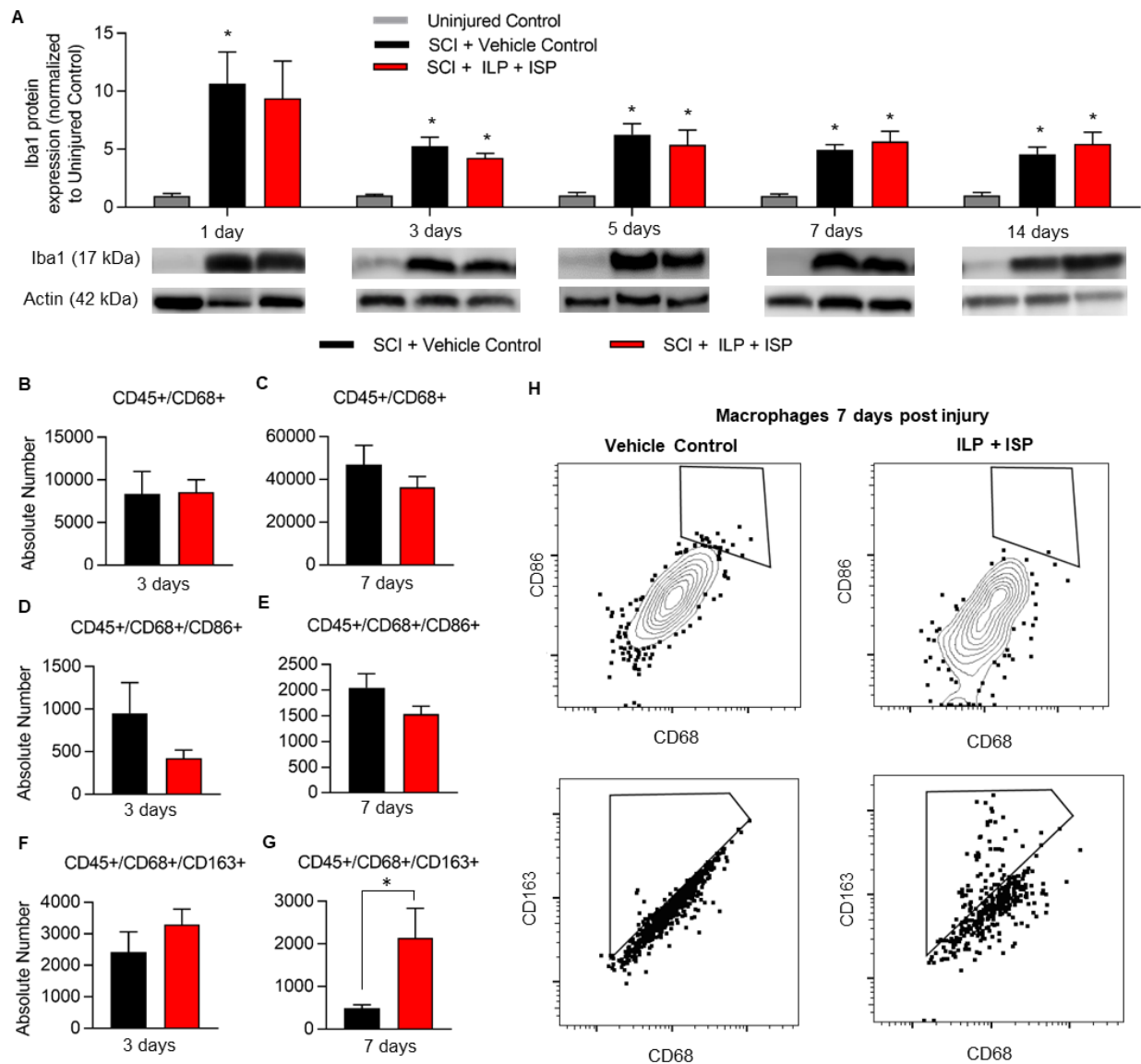
Figure 4.2: Inhibition of LAR and PTPσ promotes an increase in the subpopulation of M2 macrophages after SCI.

(A) Western blot analysis of Iba1 protein expression at 1, 3, 5, 7 and 14 days following SCI revealed no apparent change in the presence of microglia/macrophage within SCI lesion. (B-C) Similarly, flow cytometric analysis of spinal cord tissue revealed no change in the total number of infiltrated macrophages (CD45+/CD68+) at 3 and 7 days post-injury between vehicle control and ILP/ISP (10µg/day) treated SCI animals. (D-E) ILP/ISP treatment resulted in a non-significant decrease in the number of CD45+/CD68+/CD86+ M1 macrophages at 3 and 7 days post-injury. (F-G) A significant increase in the number of CD45+/CD68+CD163+ M2 macrophages was observed at 7 days post-injury in ILP/ISP treated animals. (H) Representative flow cytometry gates are depicted. N = 4-6 animals/group/time-point. The data show mean ± SEM, *p<0.05, One Way ANOVA (A), Student *t*-test (B-G).

Figure 4.3: Modulation of LAR and PTPσ attenuates pro-inflammatory cytokines while elevating anti-inflammatory mediators in SCI.
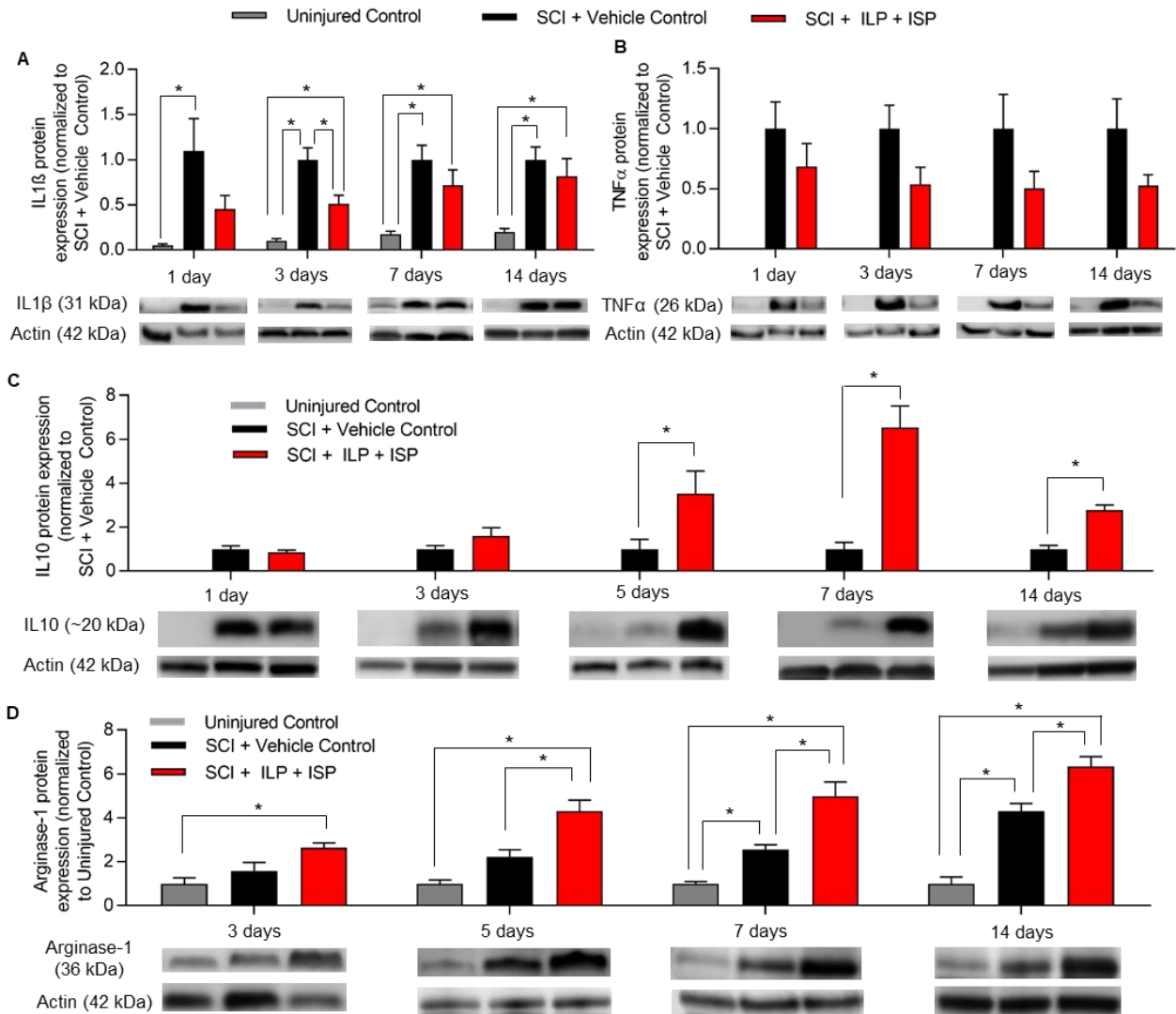
Figure 4.3: Modulation of LAR and PTPσ attenuates pro-inflammatory cytokines while elevating anti-inflammatory mediators in SCI.

(A) Western blot analysis of IL-1β protein expression at 1, 3, 7 and 14 days post-SCI showed a significant increase in IL-1β at 1 day post-SCI that persisted for up to 14 days after injury. ILP and ISP co-treatment attenuated this upregulation but was only statistically significant at the 3-day time-point post-SCI. (B) TNFα protein expression was also significantly upregulated at 1, 3, 7 and 14 days post-SCI compared to uninjured control. ILP and ISP treatment reduced TNFα levels; however, the reduction was not statistically significant. (C-D) Western blot analysis of IL-10 and Arginase-1 protein at various time-points showed that ILP and ISP co-treatment significantly increased both factors at 5, 7 and 14 days post-SCI compared to vehicle treatment. N = 4-6 animals/group/time-point. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

4.4.1.3 *T cells:* Given that inhibition of LAR and PTPσ promoted a remarkable IL-10 response, we next studied infiltrating regulatory T (T$_{reg}$) lymphocytes that also produce IL-10 in the injured spinal cord. Our flow cytometric analysis showed no apparent change in the number of CD45+/CD3+/CD4+ helper T cells with ILP/ISP treatment at both 3 and 7 days post-injury (Fig. 4.4A-B, (N=5 animals/group), $p < 0.05$, Student *t* test). Western blot analysis also confirmed our flow cytometry data showing no change in the overall number of CD4+ helper T cells between ILP/ISP and vehicle treated animals at 7 and 14 days post-injury (Fig. 4.4I, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). However, similar to macrophages, blockade of LAR and PTPσ promoted a phenotype change in T cells. ILP/ISP treatment led to a significant reduction in the number of IFNγ expressing effector T cells (CD45+/CD3+/CD4+/IFNγ+) at 7 days post-injury (Fig. 4.4C-D, $p < 0.05$, Student *t* test) while promoting a significant increase in IL-10 expressing T$_{reg}$ cells (CD45+/CD3+/CD4+/IL10+) at 3 days post-injury (Fig. 4.4E-F, $p < 0.05$, Student *t* test). Moreover, our complementary Western blot analysis also identified a significant increase in forkhead box P3 (FOXP3) protein expression, a marker associated with IL-10 expressing T$_{reg}$ cells, at both 7 and 14 days post-injury (Fig. 4.4J, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Interestingly, our immunohistochemical analysis of spinal cord tissue at 7 days post-SCI confirmed that both CD11b microglia/macrophages (Fig. 4.5A-F) and CD3 T cells (Fig. 4.5G-L) contribute to IL-10 expression following SCI, and this expression was elevated in ILP/ISP treated animals.

Figure 4.4: ILP and ISP promotes a phenotypic switch in helper T cells towards a T_reg phenotype.
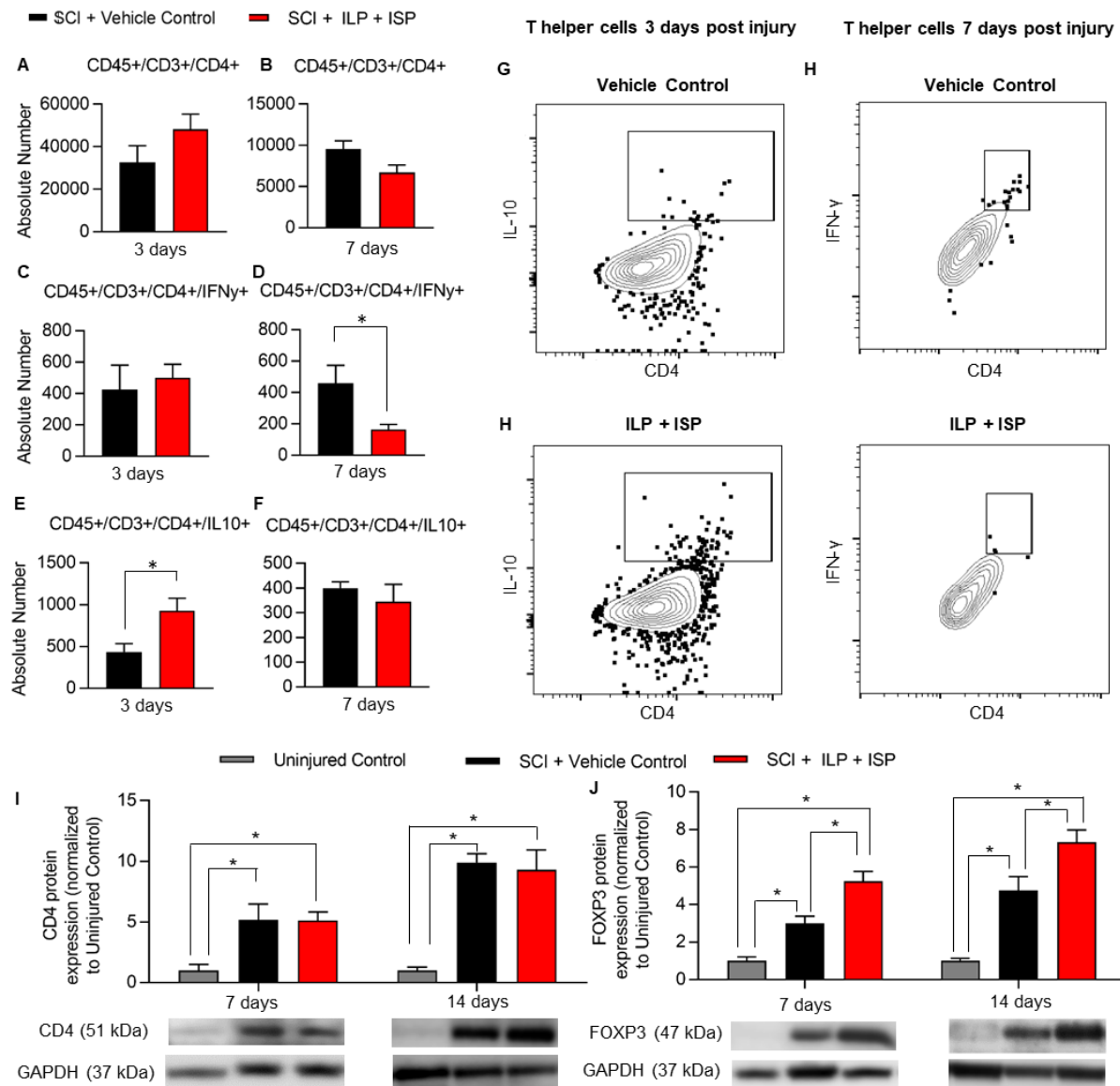
Figure 4.4: ILP and ISP promotes a phenotypic switch in helper T cells towards a T_reg phenotype.

(A-B) Flow cytometric assessment revealed no apparent difference in the overall infiltration of helper T cells (CD45+/CD3+/CD4+) in the injured spinal cord at 3 and 7 days post-injury between vehicle and ILP/ISP treated animals. (C-D) However, ILP/ISP treated animals exhibited a significant decrease in the number of effector T cells (CD45+/CD3+/CD4+/IFNγ+) at 7 days post-injury. (E-F) A significant increase in the total number of regulatory T cells (CD45+/CD3+/CD4+/IL10+) was observed at 3 days post-injury in ILP/ISP treated animals. (G-H) Representative flow cytometry gates are shown. (I) Western blot analysis showed upregulation of CD4 protein expression, at 7 and 14 days post-SCI compared to uninjured control group confirming infiltration of helper T cells in the injured spinal cord. Confirming our flow cytometry, ILP and ISP had no apparent effect on the overall protein expression of CD4. (J) However, ILP and ISP significantly increased FOXP3 protein expression, a marker of regulatory T cells, at both 7 and 14 days post-SCI compared to SCI vehicle control. Western blot results have been normalized to the actin loading control prior to subsequent normalization to the control values. The data show mean ± SEM, *$p<0.05$, Student T-test (A-F), One Way ANOVA (I-J), N=4-6/group.

Figure 4.5: Microglia/Macrophages and T cells contribute to IL-10 expression after SCI.
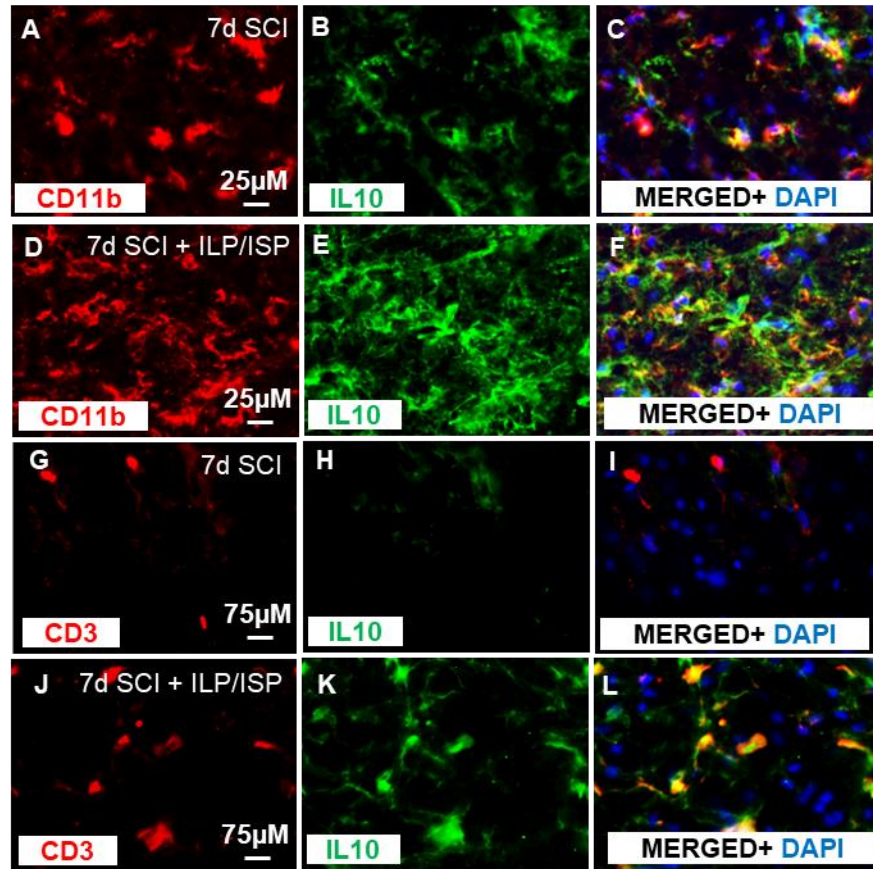
(A-L) Immunohistochemistry on spinal cord tissue confirmed an increase in IL-10 expression in ILP/ISP treated animals at 7 days following injury compared to vehicle treated animals. IL-10 expression was confirmed to be expressed in both CD11b+ macrophages/microglia (A-F) and CD3+ T cells (G-L).

4.4.2 CSPGs Negatively Modulate Microglia Responses by Signaling through LAR and PTPσ Receptors and Activation of the Rho/ROCK Pathway

Our SCI studies demonstrated that inhibition of CSPGs receptors, LAR and PTPσ, promoted an M2 phenotype. Thus, we next dissected the role and mechanisms of CSPGs in regulating the microglia response and whether LAR and PTPσ mediates CSPG effects using a direct *in vitro* primary microglia culture model. First, we confirmed that microglia express both LAR and PTPσ (Supplementary Fig. 4.5A-F). Next, we determined the direct effects of CSPGs on several aspects of microglia functions including phagocytosis, nitrite production, cytokine release, and mobilization in the various microglia phenotypes including resting (M0), classically activated (M1), and alternatively activated (M2). Microglia were polarized to an M1 phenotype by co-treatment of IFNγ (50ng/ml) and TNFα (40ng/ml) or an M2 phenotype by IL-10 (10ng/ml) treatment (Fig. 4.6A-C). M1 polarization was confirmed by induced expression of CD86 (Fig. 4.6D-F) and nitric oxide) (Fig. 4.6J, N= 5 of independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*) that was absent in M0 (untreated microglia) or M2 microglia. Similarly, M2 microglia polarization was confirmed by an increase in the mannose receptor (Fig. 4.6G-I) and IL-10 production (Fig. 4.6K, N= 5 of independent cultures $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*), two known markers for the M2 dominant phenotype (Miron et al. 2013).

Figure 4.6: Polarization of Primary Microglia Cultures to an M0, M1, or M2 phenotype.
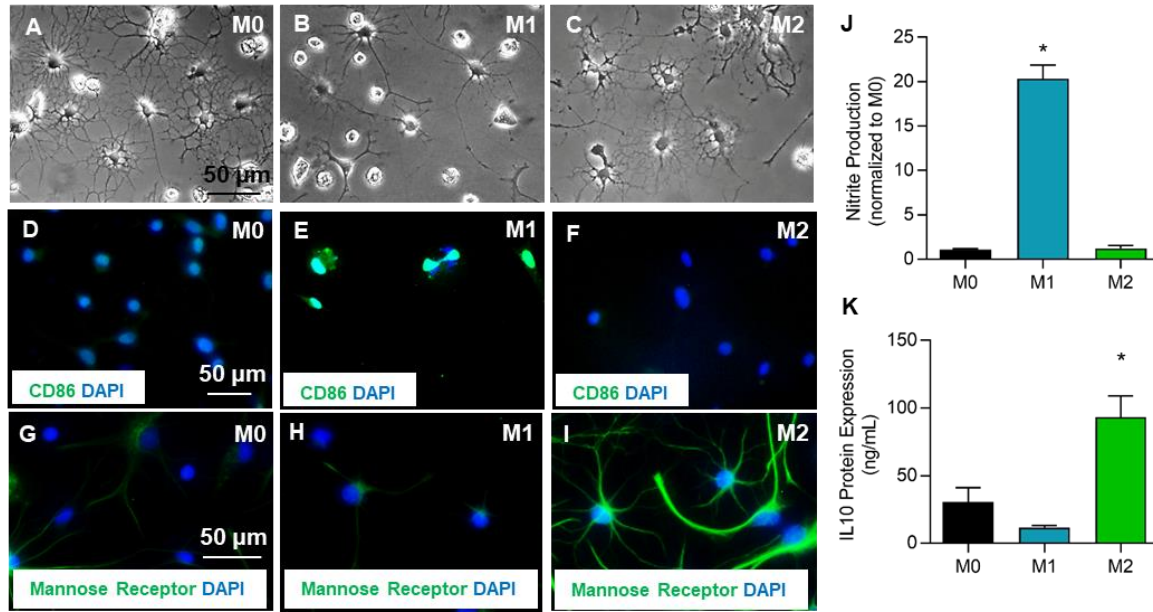
(A-C) Primary microglia were polarized to M1 through IFNγ and TNFα treatment or M2 through IL-10 treatment. M1 polarization was confirmed by induced expression of CD86 (D-F) and release of nitrite (J). Increased expression of mannose receptor (G-I) and IL-10 (K) were used to confirm M2 polarization. N = 5 independent experiments. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

4.4.2.1 *Microglia Phagocytosis:* In cultures of M0, M1 and M2 polarized microglia, we first analyzed the effects of CSPGs on modulating microglia phagocytic ability (Fig. 4.7A). M2 microglia/macrophages are responsible for the clearance of degenerating cells and myelin debris in SCI, and thereby contribute to tissue repair (Miron and Franklin 2014). Green fluorescent beads were pre-opsonized with serum and then added to microglia grown on PDL or PDL + CSPGs substrate (Fig. 4.7B-C). Presence of CSPGs significantly decreased M0 phagocytosis by 29% which was overcome by both ILP/ISP treatment and degradation of CSPGs with ChABC (Fig. 4.7A, N=3-4 independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Interestingly, treating microglia with the ROCK inhibitor Y-27632 (10μM) was also able to attenuate CSPG effects on microglial phagocytosis indicating a Rho/ROCK mediated mechanism. Notably, in M0 "resting" microglia, ILP/ISP treatment itself significantly increased microglia phagocytosis compared to PDL control group. TAT and IMP control peptides did not appear to have any modulatory effect on microglia phagocytosis on their own. M1 microglia showed reduced phagocytosis ability compared to M0 microglia, but CSPGs did not further decrease their phagocytic ability. Similar to M0 microglia, ILP/ISP treatment significantly promoted M1 microglia phagocytosis in both PDL and PDL+CSPGs treated cells suggesting the overall inhibitory role of LAR and PTPσ in phagocytosis independent of CSPGs. Our analysis revealed that M2 polarization significantly enhanced the ability of microglia for phagocytosis as compared to M1 counterparts in the baseline PDL condition. Exposure to CSPGs non-significantly decreased phagocytosis ability of M2 microglia as compared to PDL condition, which was entirely reversed by ILP/ISP treatment. Interestingly, similar to M0 and M1 microglia, ILP/ISP treatment promoted the ability of M2 microglia for phagocytosis in the presence of CSPGs. However, in contrast to

M0 and M1 microglia, ILP/ISP treatment on its own had no apparent effects on the baseline level of phagocytosis in M2 microglia in the PDL condition without CSPGs.

4.4.2.2 *Assessment of microglial phenotypes:* We further investigated whether CSPGs induce an M1 pro-inflammatory state in microglia. To this end, we studied nitrite production of microglia as an indicator of the M1 phenotype (Miron and Franklin 2014). M0 and M2 microglia released rather low levels of nitrite, which remained unaffected by the presence of CSPGs indicating that CSPGs themselves do not induce an M1 state in M0 "resting" or M2 microglia (Fig. 4.7C, N=3-4, $p <$ 0.05, One Way ANOVA, Holm–Sidak *post hoc*). However, exposure of M1 cells to CSPGs exacerbated their nitrite production compared to M1 microglia grown on PDL. Interestingly, this effect was not blocked by ILP and ISP treatment or Y-27632; while ChABC did block CSPGs induced nitrite production in M1 cells. These results indicate that a CSPG dependent mechanism exists for nitrite release in M1 microglia that is not mediated by LAR and PTPσ or Rho/ROCK pathways. TAT and IMP control peptides also had no apparent effect on microglia nitrite levels.

We then examined whether CSPGs modulate cytokine release by microglia. We studied IL-1β and IL-10 representing the M1 and M2 dominant phenotype, respectively. Overall, there was no apparent change in IL-10 or IL-1β expression in M0 "resting" microglia when grown on PDL or PDL+CSPGs substrate (Fig. 4.7D-E, N=3-4 independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). CSPGs did however non-significantly decrease IL-10 expression in M2 microglia which was brought to control levels with ILP/ISP, Y-27632 and ChABC treatment. Conversely, IL-1β expression was non-significantly increased when M1 microglia were exposed to CSPGs substrate. Given the involvement of the Rho/Rock pathway in CSPG effects, we sought to determine whether LAR and PTPσ receptors mediate these effects in microglia

cultures. M1 microglia grown on CSPGs substrate had a 4-fold increase in Rho activity that was significantly reduced by ILP/ISP (Fig. 4.7G, N=3 independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). These findings collectively identify the Rho/ROCK pathway as a downstream mediator of CSPGs and LAR and PTPσ signaling.

4.4.2.3 *Microglial mobilization:* Mobilization of resident microglia to the site of injury is an important aspect of neuroinflammatory processes. Therefore, we investigated whether CSPGs influence the ability of microglia for mobilization using C5a as a chemoattractant in a transwell assay (Miller and Stella 2009). We quantified the number of microglia which crossed over PDL or PDL+CSPGs coated inserts in a 16-hour period. CSPGs significantly decreased microglia mobilization, which was partially yet significantly attenuated by both ILP/ISP and Y-27632 treatments in a comparable manner (Fig. 4.7F, N=3 independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Interestingly, degradation of CSPGs with ChABC was able to block their CSPGs inhibitory effects on microglia mobilization entirely suggesting involvement of other mechanisms. Taken together, these data indicate that CSPGs modulate several facets of microglia activity by signaling through LAR and PTPσ and activation of the Rho/ROCK pathway.

Figure 4.7: CSPGs modulate microglia phagocytosis, migration and nitrite production which is partially mediated through LAR and PTPσ signaling and Rho activation.
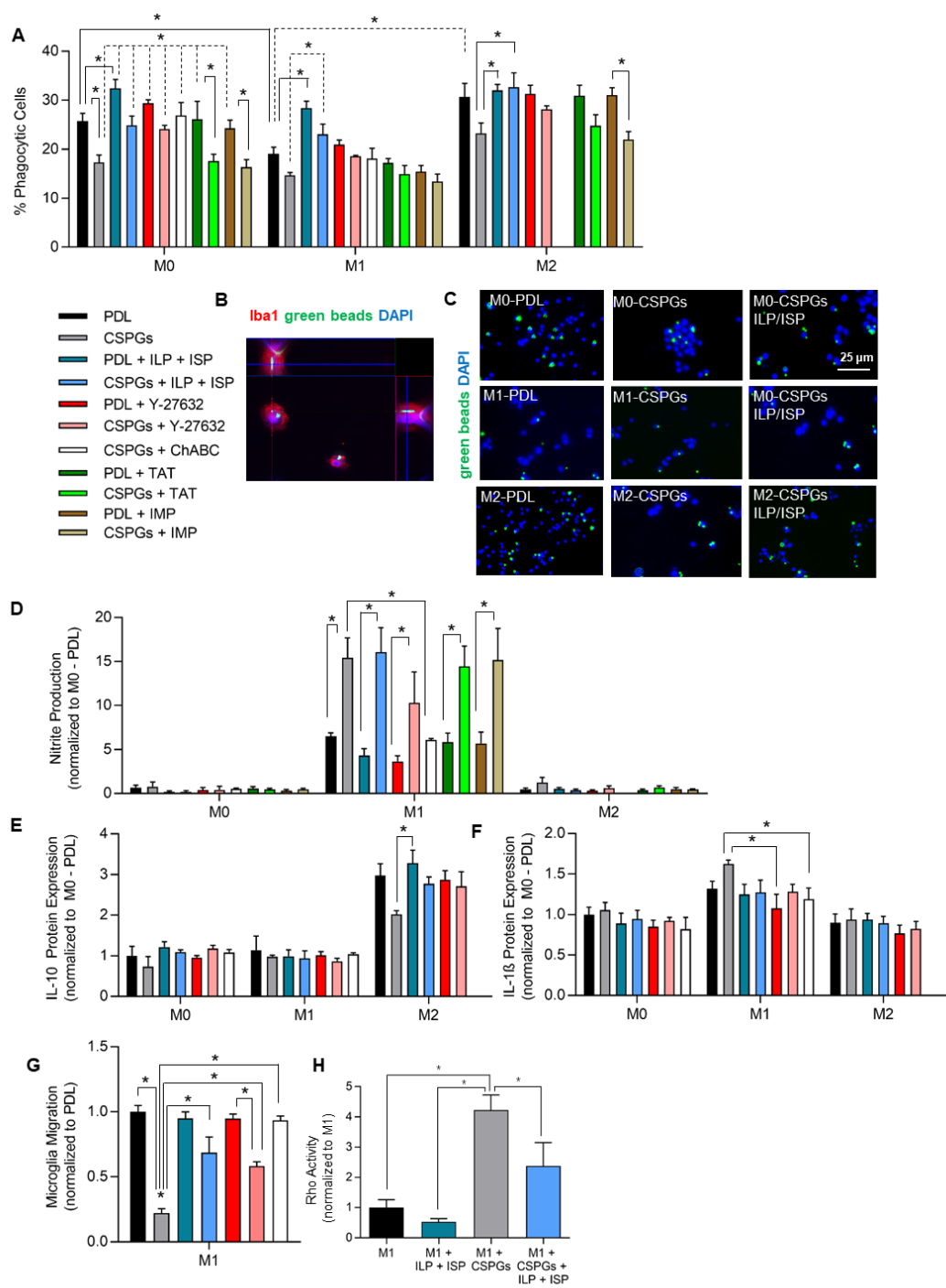
Figure 4.7: CSPGs modulate microglia phagocytosis, migration and nitrite production which is partially mediated through LAR and PTPσ signaling and Rho activation.

(A-B) Microglia phagocytosis was assessed. Success of phagocytosis was verified by intracellular detection of green fluorescent beads in microglia (Iba-1+) through Z-stack imaging. M1 microglia (TNFα + IFNγ treated) showed a reduced ability for phagocytosis. CSPGs reduced phagocytosis in microglia, which was attenuated and even promoted with ILP/ISP, inhibition of ROCK by Y-27632, or ChABC treatment but not by TAT or IMP control peptides. (C) Representative images of phagocytosis by M0, M1 and M2 microglia are depicted for PDL, CSPGs and CSPGs+ILP/ISP conditions. (D) Nitrite production was exacerbated and significantly increased in M1 microglia when exposed to CSPG. This effect was not blocked by ILP/ISP, Y-27632, IMP, or TAT treatment but was blocked by ChABC degradation of CSPGs. IL-10 (E) and IL-1β (F) release was assessed in microglia 2 days after plating onto PDL or PDL+CSPGs substrate. CSPGs reduced IL-10 expression in M2 microglia while had no significant effect on IL-1β release. (G) CSPGs also significantly limited microglia migration which was overcome by ILP/ISP, Y-27632, and ChABC treatment. (H) RhoA activity was assessed by G-LISA in microglia demonstrating a significant increase in Rho activity when microglia were exposed to CSPGs substrate. ILP and ISP treatment significantly decreased Rho activity. The data show the mean ± SEM, *p<0.05, One Way ANOVA, N=3-5/group. N = 3-5 independent experiments. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

4.4.3 M2 Microglia Promote Oligodendrogenesis by NPCs through an IL-10 Mediated Mechanism

Emerging evidence has implicated M2 inflammatory responses and specifically increased IL-10 release in enhancing endogenous repair mechanisms following CNS injury (Bollaerts et al. 2017; Miron and Franklin 2014; Miron et al. 2013; Xu et al. 2017). M2 microglia have been shown promote oligodendrocyte differentiation and myelination in lysolecithin demyelinating mouse models (Miron et al. 2013). Interestingly, previous findings by our group and others have revealed that inhibition of CSPGs can also enhance oligodendrogenesis in SCI and demyelinating conditions (Karimi-Abdolrezaee et al. 2012; Lau et al. 2012; Keough et al. 2016). Here, we investigated whether the immunomodulatory benefits of inhibiting LAR and PTPσ in promoting an M2 phenotype and IL-10 production can foster oligodendrocyte differentiation. We addressed this question using primary cultures of adult spinal cord derived NPCs.

First, we assessed whether an increase in the production of IL-10 can directly impact the regenerative response of NPCs by utilizing recombinant IL-10 *in vitro*. We treated NPCs with recombinant IL-10 and studied their proliferation and differentiation patterns. Assessing the percentage of BrdU+ proliferating NPCs under various concentration of IL-10 showed no apparent effect on NPC proliferation (Fig. 4.8A-C, N = 5 independent cultures, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). However, IL-10 had a modulatory effect on the differentiation pattern of NPCs. IL-10 treatment significantly reduced the number of NPC derived GFAP+ astrocytes while increasing Olig2+ oligodendrocytes at 100ng and 200ng, which was blocked using 0.8ug and 1.6ug of IL-10 neutralizing antibody, respectively (Fig. 4.8D-K, N =4, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). These direct analyses indicate that availability of IL-

10 can promote NPCs differentiation along an oligodendrocyte lineage with no apparent effects on their proliferation.

Next, we focused on dissecting the impact of IL-10 derived from M2 polarized microglia on the regenerative response of spinal cord NPCs. Microglia were polarized to an M1 or M2 phenotype as previously described. Two days following microglial polarization, microglia conditioned media (MCM) was harvested and added to NPC cultures for proliferation and differentiation assessments (Fig. 4.9A). To assess microglial derived IL-10 effects on NPCs *in vitro,* we blocked IL-10 using a neutralizing antibody. To estimate the IL-10 protein concentration in the MCM of M2 microglia, our ELISA analysis determined a concentration of 100ng/ml for IL-10 in MCM (N = 5, Fig. 4.6K). Therefore 0.8µg/ml of IL-10 neutralizing antibody was used as this dose was shown to sufficiently block the effects of 100ng/ml of IL-10 on NPCs oligodendrocyte differentiation *in vitro* (Fig. 4.8D-E). Using these parameters, we found that M2 MCM enhances the overall proliferation of NPCs whereas M1 MCM reduces their capacity for proliferation (Fig. 4.9B, N = 4, $p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Nonetheless, this effect was found to be IL-10 independent indicating that other factors associated with M2 microglia attribute to the increase in NPC proliferation. Our NPC differentiation studies showed that M2 MCM also promotes a significant increase in oligodendrogenesis of NPCs compared to M0 and M1 MCM (Fig. 4.9C-N, N = 4). Interestingly, this effect was partly attributed to M2 derived IL-10 since an IL-10 neutralizing antibody was able to reverse some of the effect. Conversely, M1 MCM significantly reduced the percentage of NPC derived oligodendrocytes compared to M0 and M2 MCM ($p < 0.05$, One Way ANOVA, Holm–Sidak *post hoc*). Astrocyte differentiation of NPCs was decreased by M2 MCM in comparison to M0 and M1 MCM

suggesting that M2 polarization supports oligodendrogenesis of spinal cord NPCs. Taken together, these data demonstrate the pivotal role of microglia in regulating endogenous cell differentiation.

Figure 4.8: IL-10 promotes oligodendrocyte differentiation of spinal cord NPCs *in vitro*.
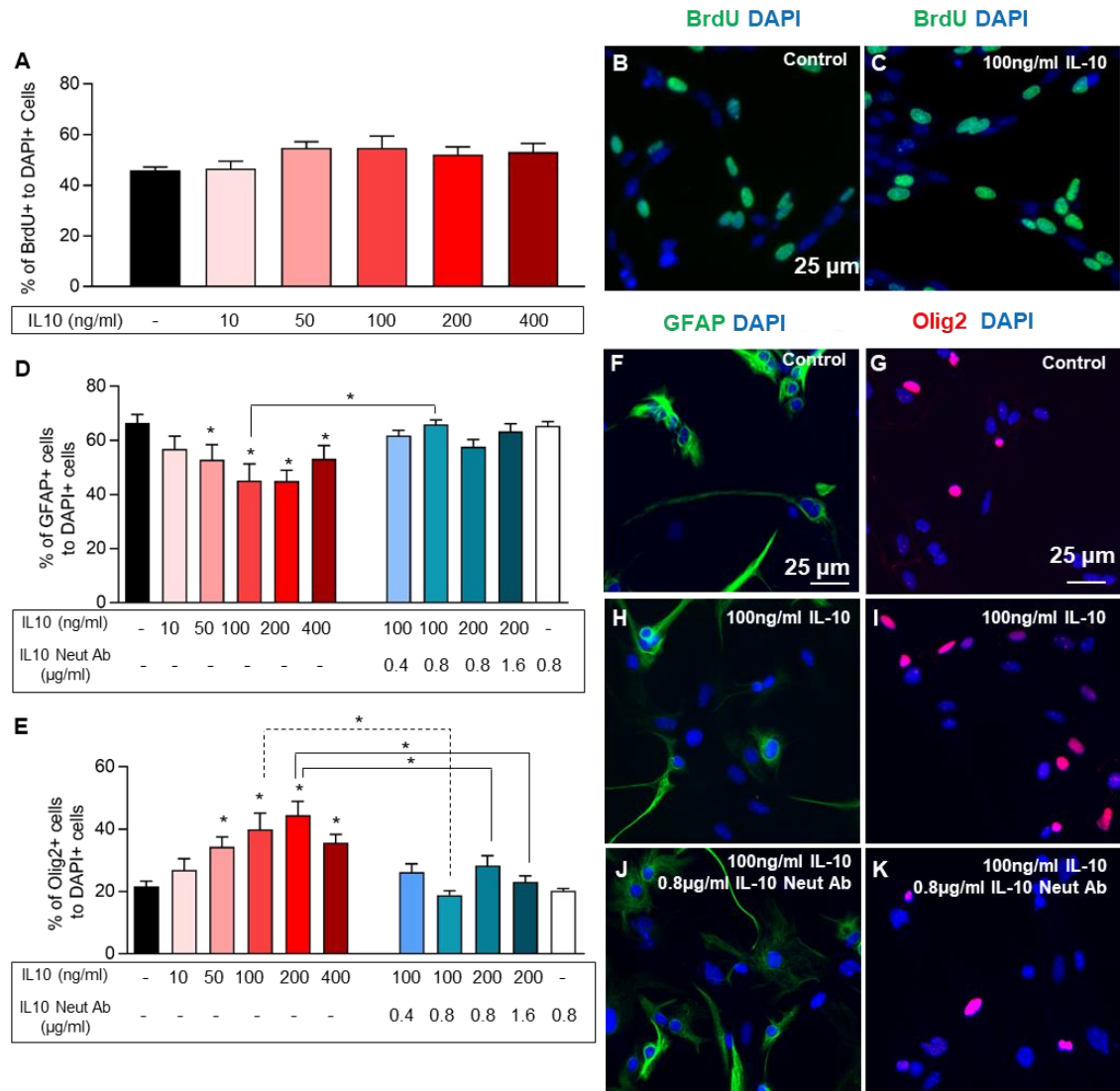
Figure 4.8: IL-10 promotes oligodendrocyte differentiation of spinal cord NPCs *in vitro*.

(A-C) Addition of recombinant IL-10 had no apparent effect on NPCs proliferation (BrdU+/DAPI+) at all doses tested. (D) However, IL-10 significantly reduced the percentage of GFAP+/DAPI+ astrocytes at 100 and 200ng/ml, (E) while increased the percentage of Olig2+ cells at 50, 100, 200 and 400ng/ml with the highest effect at 200ng. This effect was significantly attenuated with IL-10 neutralizing antibody. Addition of 0.8µg/ml of IL10 neutralizing antibody effectively blocked 100ng/ml of IL-10 on astrocyte and oligodendrocyte differentiation of NPCs. (F-K) Representative images of NPC differentiation assessment are shown. N = 3-5 independent experiments. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

Figure 4.9: M2 microglia promote oligodendrocyte differentiation of spinal cord NPCs through IL-10.
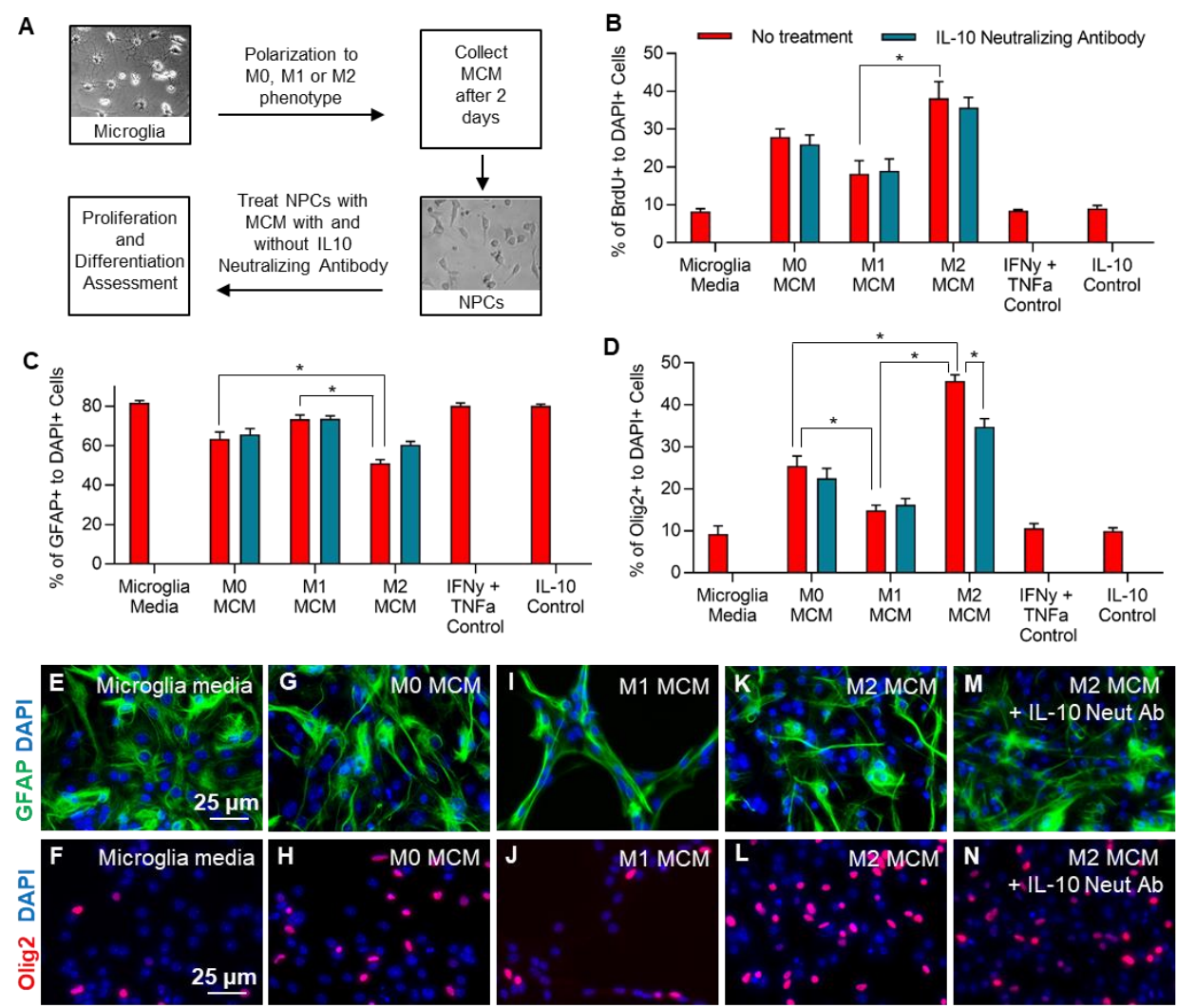
Figure 4.9: M2 microglia promote oligodendrocyte differentiation of spinal cord NPCs through IL-10.

(A) To assess the effects of microglia effects on NPC proliferation and differentiation, MCM was collected from microglia 2 days following polarization. This media was then transferred to NPC cultures to assess proliferation and differentiation. (B) MCM derived from M2 microglia significantly promoted NPC proliferation compared to M1 MCM. IL-10 neutralizing antibody had no effect on the overall proliferation of NPCs by M2 MCM suggesting this effect was not mediated through IL-10. (C) A significant decrease was observed in the percentage of GFAP+/DAPI+ astrocytes when NPCs were exposed to M2 MCM (K) compared to both M0 (G) and M1 (I) MCM. (D) M2 MCM (L) significantly increased the percentage of Olig2+/DAPI+ cells compared to both M0 (F) and M1 (J) MCM. Additionally, M1 MCM significantly reduced the percentage of Olig2+ cells compared to both M0 and M2 MCM. (M-N) The effect of M2 MCM was significantly reduced by IL-10 neutralizing antibody. N = 3-5 independent experiments. The data show the mean ± SEM, *p<0.05, One Way ANOVA.

4.4.4 Inhibition of LAR and PTPσ has no effects on CSPGs Expression and Scar Formation in SCI

Lastly, we asked whether LAR and PTPσ control CSPGs expression and scar formation following injury through self-regulatory mechanisms. Immunoblotting for GFAP and CSPGs at 1, 3, 5, 7, and 14 days following SCI showed no significant difference in GFAP and CSPGs protein expression between ILP/ISP and vehicle treated animals at any examined time-point (N=4-6 animals/group, Fig. 4.10A, 10C). Similarly, our immunohistochemical measurement of GFAP and CSPGs within the SCI lesion at 7 days (data not shown) and 28 days post-injury suggested no significant difference between vehicle and ILP/ISP treatment groups (Fig. 4.10B, 10D, N=5-6 animals/group). As expected SCI induced an anticipated significant increase in CSPGs and GFAP levels compared to uninjured baseline at all time-points post-injury, however, ILP/ISP treatment did not alter their expression. Altogether, these findings suggest that LAR and PTPσ are not apparently involved in astrocytic scar formation and CSPG deposition in the injured spinal cord.

Figure 4.10: Blocking LAR and PTPσ receptors has no effect on formation of astrocytic scar and CSPGs following SCI.
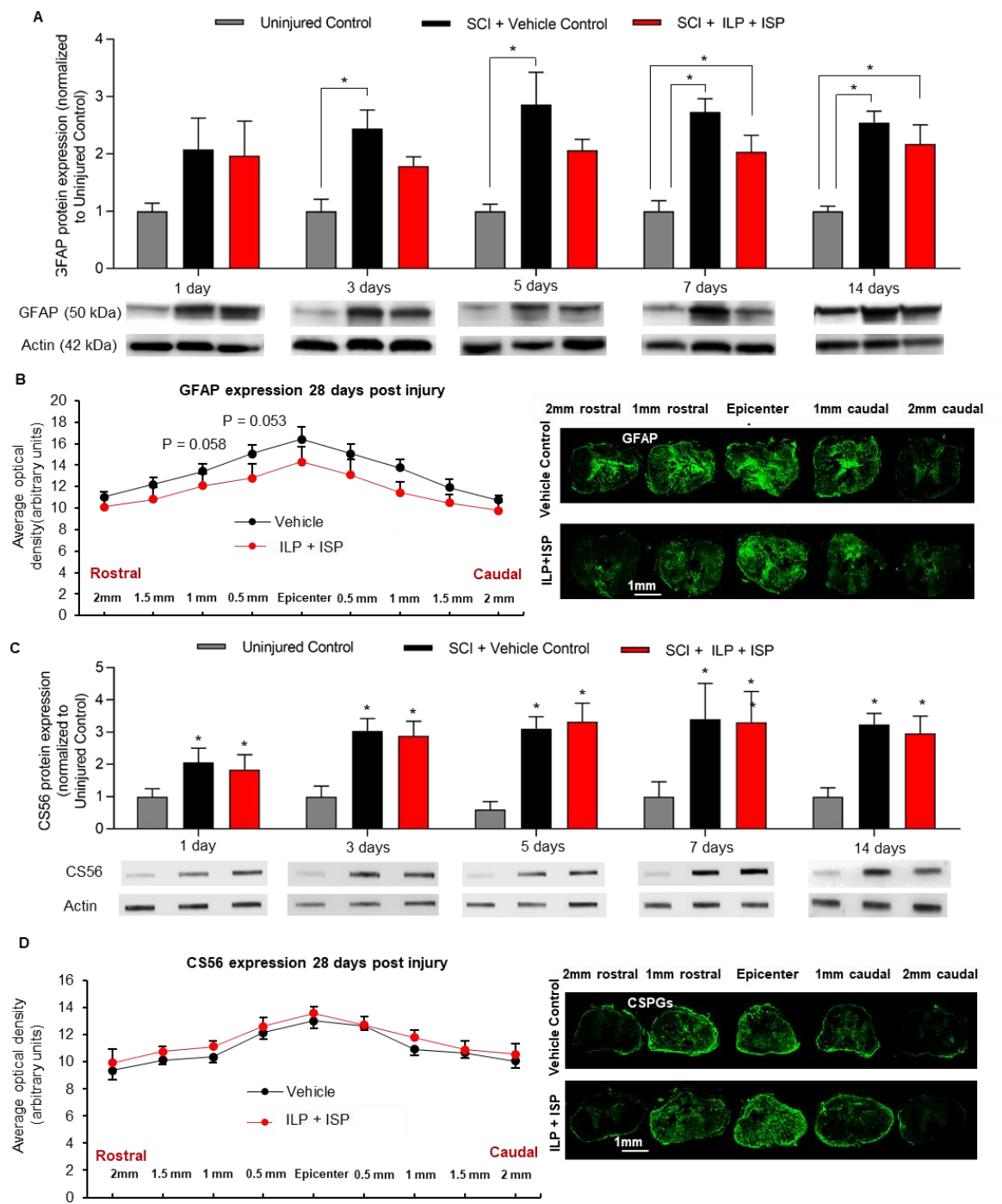
Figure 4.10: Blocking LAR and PTPσ receptors has no effect on formation of astrocytic scar and CSPGs following SCI.

(A) To study scar formation under ILP and ISP treatment, we examined GFAP protein expression by Western blotting at 1, 3, 5, 7 and 14 days post-injury. As anticipated, GFAP protein expression was significantly increased following injury compared to uninjured animals. However, ILP and ISP treatment had no significant effect on the expression of GFAP at any time-points after SCI. (B) Using immunohistochemistry, we also studied astrocytic scar in SCI lesion. Our quantitative immunodensity analysis in the injured spinal cord tissue at the chronic 28 days post-SCI also showed no significant differences in the levels of GFAP under ILP/ISP treatment compared to vehicle SCI control group. (C) Slot blot analysis of CS56 expression at 1, 3, 5, 7 and 14 days post-SCI demonstrated a significant increase CSPGs following injury. Similar to GFAP, ILP and ISP treatment had no effect on the deposition of CSPGs following injury. (D) CS56 expression was additionally measured using immunohistochemistry at different distances to injury epicenter at 28 days post-SCI. ILP/ISP treatment showed no significant differences in the levels of CS56 compared to vehicle SCI control group. The data show the mean ± SEM, $*p<0.05$, $**p<0.001$, One Way ANOVA (A, C), Two Way ANOVA (B-D), N=4-6/group.

4.5 Discussion

4.5.1 *Overview of findings:* In the present study, we have identified novel inhibitory mechanisms for CSPGs and their specific signaling receptors, LAR and PTPσ, in modulating the immune response after SCI. In a clinically relevant model of compressive/contusive SCI in rat, we demonstrate that modulation of CSPG signaling with ILP and ISP treatment drives an anti-inflammatory and pro-regenerative immune response that is characterized by increased populations of M2 microglia/macrophages and T regulatory cells within the injured spinal cord. This cellular response was associated with an overall decrease in pro-inflammatory markers including IL-1β and TNFα and an increase in regulatory mediators such as IL-10, Arginase-1 and FOXP3. Our complementary *in vitro* studies in primary microglia revealed that while CSPGs do not seem to be an inducer of the M1 phenotype in microglia *per se*; their presence in the milieu of M1 microglia promotes and/or maintains their pro-inflammatory phenotype. We demonstrate that CSPGs promote production of M1 markers IL-1β and nitrite in microglia. Interestingly, while the CSPG-induced increase in IL-1β was attenuated by ILP/ISP, nitrite production was not mediated by LAR and PTPσ signaling. This evidence suggests that CSPGs seem to regulate nitrite production in M1 microglia through other mechanisms, which needs further elucidation. Interestingly, the presence of CSPGs also supressed IL-10 release by M2 polarized microglia, which was ameliorated by inhibition of LAR and PTPσ signaling. Importantly, we provide novel direct evidence that CSPGs, through LAR and PTPσ activation, hinder the ability of microglia for phagocytosis and mobilization, and suppress their potential for promoting oligodendrocyte differentiation of NPCs. Mechanistically, we demonstrate that LAR and PTPσ mediate the effects of CSPG on microglia through RhoA activation and the ROCK pathway. Altogether, our parallel findings in SCI and microglia culture have uncovered, for the first time, a negative

immunomodulatory role for CSPGs and the LAR/PTPσ axis that can contribute to the poor regenerative response after SCI. Thus, we propose that targeting LAR and PTPσ represents a potential immunotherapy strategy for SCI.

4.5.2 *Inhibition of LAR and PTPσ positively regulates inflammatory processes after SCI:*

Neuroinflammation is a complex process in SCI that involves several cell types including resident microglia and infiltrating leukocytes (Popovich, Wei, and Stokes 1997; Hausmann 2003). The first phase of neuroinflammation involves recruitment of resident microglia and neutrophils. Neutrophils are the first leukocyte population to enter the spinal cord after insult (Taoka et al. 1997; Sroga et al. 2003; Neirinckx et al. 2014). Neutrophils are recruited to the injury site within 6 hours after injury and their numbers peak within 24 hours (Taoka et al. 1997; Neirinckx et al. 2014). They are generally thought to exacerbate the injury process by damaging neurons, glial cells and endothelial cells, and through their release of toxic ROS and proteases (Nguyen, O'Barr, and Anderson 2007; Dinkel, Dhabhar, and Sapolsky 2004). Here, we demonstrate the ability of ILP and ISP treatment to limit the overall acute infiltration of neutrophils following SCI. Reducing neutrophil infiltration is shown to attenuate the extent of tissue damage after SCI (Kang et al. 2011; Neirinckx et al. 2014; Dumont et al. 2001) in which may have contributed to the functional recovery observed after targeting LAR and PTPσ in previous studies (Lang et al. 2015; Fisher et al. 2011).

The second phase of inflammation involves the recruitment of macrophages to the injury site (David and Kroner 2011; Miron and Franklin 2014). Macrophage infiltration is necessary following injury as supressing the M2 population chronically by ablating the macrophage cellular pool is associated with worse outcomes in mice SCI (Shechter et al. 2009). The impact of resident

microglia and infiltrating macrophages on the repair process is determined by their activation state; being classically activated M1, or alternatively activated M2 (David and Kroner 2011; Kigerl et al. 2009). Initially, there is a balanced ratio between pro-inflammatory M1 and pro-regenerative M2 microglia/macrophages in the SCI lesion. However, as injury progresses this landscape switches predominantly towards an M1 phenotype forging cell death and tissue degeneration (Kigerl et al. 2009; Takahashi et al. 2003; David and Kroner 2011).

Our immunophenotyping studies revealed that disruption of LAR and PTPσ signaling by ILP/ISP promotes M2 macrophages as well as an overall increase in the expression of key pro-regenerative immune mediators, IL-10 and Arginase-1. Our findings are in agreement with recent studies where ChABC treatment also induced an overall anti-inflammatory response following SCI (Bartus et al. 2014; Didangelos et al. 2014). The M2 inflammatory response is generally associated with improved outcomes after CNS pathology (Miron et al. 2013). Transplantation of exogenous M2 cells promotes functional recovery following SCI indicating their ability to foster a regenerative program in the injured spinal cord (Rapalino et al. 1998).

4.5.3 *M2 microglia promote oligodendrocyte differentiation of NPCs through IL-10 release:* The importance of microglia and their inflammatory phenotype in regulating endogenous cell differentiation is becoming increasingly more appreciated in CNS repair (Kokaia et al. 2012). For example, increase in pro-inflammatory cytokines such as TNFα and IL-6 negatively affect hippocampal neurogenesis in the LPS-treated brain whereas anti-inflammatory cytokines such as insulin-like growth factor-1 promote cell renewal (Butovsky et al. 2006; Monje, TOda, and Palmer 2003). Here, we provide direct evidence that inhibition of LAR and PTPσ promotes M2 microglia that are potentially beneficial for NPC proliferation and oligodendrocyte differentiation.

Interestingly, our *in vitro* NPC studies unraveled that IL-10 is critically important for M2 mediated increase in oligodendrogenesis but dispensable for NPCs proliferation. Importantly, in primary cultures of NPCs we previously established a direct role for CSPGs in restricting oligodendrogenesis through LAR and PTPσ dependent mechanisms without the presence of microglia or IL-10 (Dyck et al. 2015). Our previous ChABC studies in SCI also signified a negative role for CSPGs in restricting the survival, proliferation and oligodendrocyte differentiation of endogenous and transplanted NPCs in rat SCI (Karimi-Abdolrezaee et al. 2012; Karimi-Abdolrezaee et al. 2010). Taken together, our new and previous findings suggest that CSPGs and LAR/PTPσ can influence oligodendrogenesis by direct signaling on NPCs and indirectly by modulation of M2 microglia and IL-10 expression. Similarly, Miron et al recently demonstrated the benefits of M2 cells in promoting maturation and differentiation of OPCs *in vitro* and in demyelinating conditions (Miron et al. 2013). This evidence collectively supports the importance of microglial response on endogenous oligodendrocyte replacement following CNS injury.

4.5.4 *Inhibition of LAR and PTPσ promotes microglia phagocytosis and mobilization:* We have provided novel evidence that CSPGs may restrict the repair process by impairing the ability of microglia for phagocytosis through LAR/PTPσ dependent mechanisms as ILP/ISP treatment was able to effectively reverse CSPG effects. Interestingly, in M1 microglia, inhibition of LAR/PTPσ also promoted phagocytosis in the absence of CSPGs suggesting that these receptors may interact with other ligands or have other functions. Nevertheless, promoting the ability of microglia for phagocytosis is beneficial for the repair process (David and Kroner 2011). It is known that impaired phagocytosis by microglia is correlated with limited tissue regeneration in traumatic CNS injuries and neurodegenerative diseases (Neumann, Kotter, and Franklin 2009; David and Kroner

2011; Kroner et al. 2014). Here, we provide evidence that suggests a plausible link between the long-lasting upregulation of CSPGs after SCI and the impaired clearance of debris in the injured spinal cord. Interestingly, we demonstrate that inhibiting LAR and PTPσ enhances microglia mobility on a CSPGs substrate. Migration of activated microglia to the site of spinal cord and brain injury is important for their contribution to the repair process including phagocytosis of debris and wound healing (Davalos et al. 2005; David and Kroner 2011). In vivo imaging of microglia has identified them as highly motile cells in their environment (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). Our data suggest that the presence of CSPGs limit microglia mobility, and LAR and PTPσ appear to partially mediate this effect as ILP/ISP treatment was able to significantly attenuate the negative effects of CSPGs on microglia mobilization. Taken together, our new findings substantiate an impact for LAR and PTPσ signaling in mediating the restrictive effects of CSPGs on microglia, and demonstrate the importance of their inhibition in harnessing the potential of microglia in supporting endogenous repair processes after SCI.

4.5.5 *CSPGs regulate microglia response through LAR and PTPσ and a Rho/ROCK mechanism:* We have unraveled that Rho activation appears to be a mechanism by which CSPGs promote a pro-inflammatory phenotype in microglia. We identified that upon exposure to CSPGs microglia elevate their Rho activity downstream to LAR and PTPσ signaling. Previous work by our group and others also identified that blocking the Rho/ROCK pathway overcomes CSPG effects in multiple cell types *in vitro*, including OPCs (Pendleton et al. 2013), NPCs (Dyck et al. 2015), and neurons (Ohtake et al. 2016; Dergham et al. 2002; Monnier et al. 2003; Duffy et al. 2009). Genetic knockout studies in primary cerebellar granule neurons revealed that both LAR and PTPσ independently mediate CSPGs ability to activate the Rho/ROCK pathway (Ohtake et al.

2016). Our previous studies in spinal cord derived NPCs also substantiated that CSPGs restrict multiple properties of NPCs by activation of the Rho/ROCK pathway (Dyck et al. 2015). We showed that CSPGs mediate their effects on NPCs through both LAR and PTPσ receptors, and therefore their co-inhibition was required to block negative effects of CSPGs. Interestingly, a ROCK inhibitor also reversed CSPGs negative effects on NPCs comparable to co-inhibition of LAR and PTPσ (Dyck et al. 2015). Notably, drugs which target the Rho/ROCK pathway have shown efficacy in promoting repair and functional recovery after SCI (Monnier et al. 2003; Duffy et al. 2009; Impellizzeri et al. 2012; Eftekharpour et al. 2016; McKerracher and Anderson 2013; Fehlings et al. 2011).

Although LAR and PTPσ receptors play an important role in CSPGs associated signaling cascade (Coles et al. 2011; Shen et al. 2009; Ohtake et al. 2016; Fry et al. 2010), CSPGs are also shown to bind to Nogo66 receptors, NgR1 and NgR3 (Dickendesher et al. 2012). Nogo66 receptors were originally identified for their role in mediating the inhibitory effects of the myelin associated inhibitor Nogo on axon regeneration in the CNS (Fournier, GrandPre, and Strittmatter 2001; Mehta et al. 2007). However, studies by Dickendesher and colleagues identified that NgR1 and NgR3 also show high affinity binding to CSPGs (Dickendesher et al. 2012). Accordingly, with inhibition of LAR and PTPσ with ILP/ISP, CSPGs may still exert their effects by signaling through other mechanisms including NgR1 and NgR3. Moreover, PTPσ and LAR have shown the affinity to interact with other ligands such as heparan sulfate proteoglycan (HSPGs) (Dyck and Karimi-Abdolrezaee 2015; Fox and Zinn 2005; Aricescu et al. 2002). Interestingly, interaction between HSPGs-PTPσ and CSPGs-PTPσ has different outcomes. HSPGs-PTPσ activation promotes axon growth and synapse formation (Coles et al. 2011; Aricescu et al. 2002), whereas CSPG-PTPσ signaling inhibits regeneration (Coles et al. 2011; Shen et al. 2009). Similarly, while LAR appears

to bind to CSPGs with high affinity (Fisher et al. 2011), it also interacts with HSPGs (Fox and Zinn 2005) and homophillically to itself (Yang et al. 2003; Yang et al. 2005). Thus, ILP/ISP therapy in our studies may have influenced interaction of LAR and PTPσ with other ligands.

4.6 Conclusion

In conclusion, we provide novel evidence that CSPGs promote a predominantly pro-inflammatory microenvironment in the injured spinal cord. LAR and PTPσ receptors mediate immunomodulatory effects of CSPGs, and their inhibition activates a supportive and regulatory immune response in SCI. Intracellularly, we show that LAR and PTPσ signaling appears to regulate microglia by activating the RhoA/ROCK pathway. Thus, our work has uncovered a key role for LAR and PTPσ in neuroinflammation after SCI, and provides novel insights into the mechanisms by which targeting CSPGs can ameliorate the untoward outcomes of SCI. Importantly, this work demonstrates the promise of ILP/ISP as a potential immunotherapy strategy for the treatment of SCI and other neuroinflammatory conditions of the central nervous system.

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4.8 Supplementary Figures

Supplementary Figure 4.1. Verification of antibody specificity for phenotypical analysis of macrophages in SCI tissues.
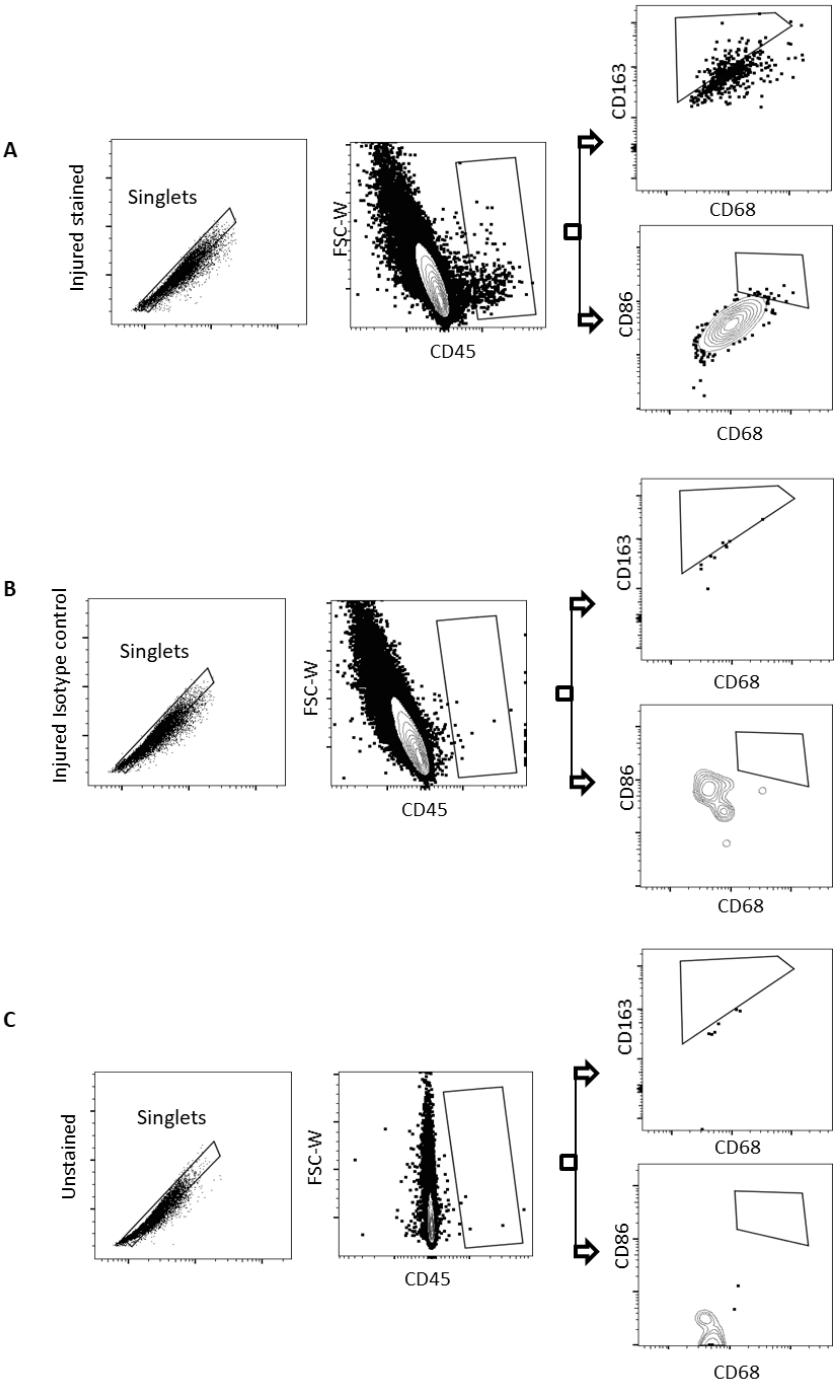
Supplementary Figure 4.1. Verification of antibody specificity for phenotypical analysis of macrophages in SCI tissues.

(A-C) Flow cytometric verification of antibody specificity was performed on SCI tissue. Isolated spinal cord immune cells were stained and gated for the detection of macrophages and their pro-inflammatory (M1, $CD45^+CD68^+CD86^+$) and pro-regenerative (M2, $CD45^+CD68^+CD163^+$) sub-populations. Results were compared with unstained and isotype control cells for each antibody analyzed using the same gating strategy. (B) A negligible number of macrophages and their subtypes were detected in the injured isotype control compared to our injured stained group confirming the specificity of antibodies used in our macrophage panel. (C) Similarly, no significant detection was observed in unstained samples analyzed with the same gating strategies.

Supplementary Figure 4.2. Verification of antibody specificity for phenotypical analysis of helper T cells in SCI tissues.
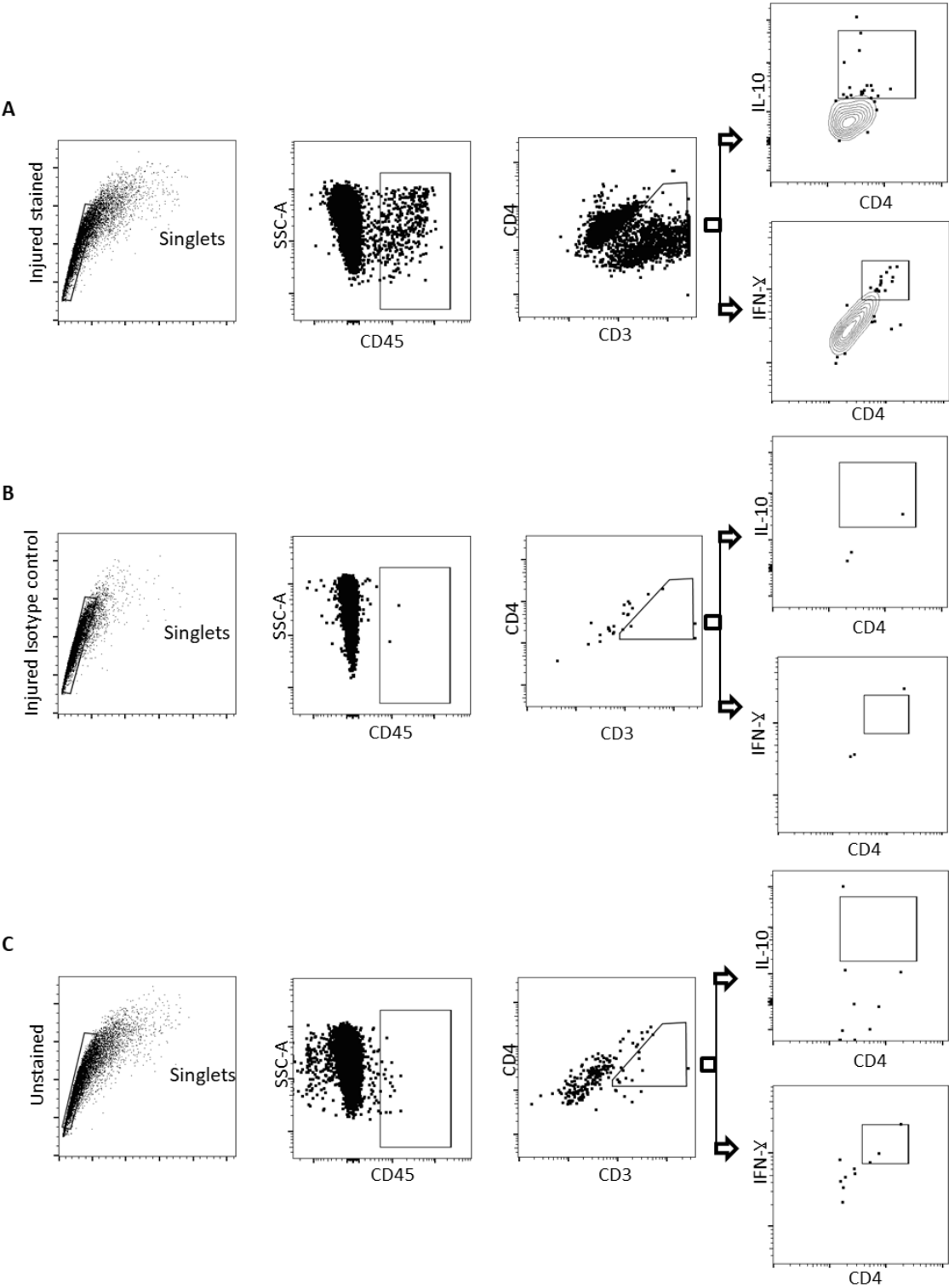
Supplementary Figure 4.2. Verification of antibody specificity for phenotypical analysis of helper T cells in SCI tissues.

(A-C) Flow cytometric verification of antibody specificity for T cell detection. Isolated spinal cord immune cells were stained and gated for the detection of helper T cells and their effector (T_{eff}, $CD3^+CD4^+IFN\gamma^+$) and regulatory (T_{reg}, $CD3^+CD4^+IL\text{-}10^+$) sub-populations. Results were compared with unstained and isotype control cells for each antibody analyzed using the same gating strategy. (B) A negligible number of T helper cells and their subtypes were detected in the injured isotype control compared to our injured stained group confirming the specificity of antibodies used in our T cell panel. (C) Similarly, no significant detection was observed in unstained samples analyzed with the same gating strategy.

Supplementary Figure 4.3: Summary of flow cytometry gating strategy used for phenotypical analysis of macrophages in injured spinal cord tissue.
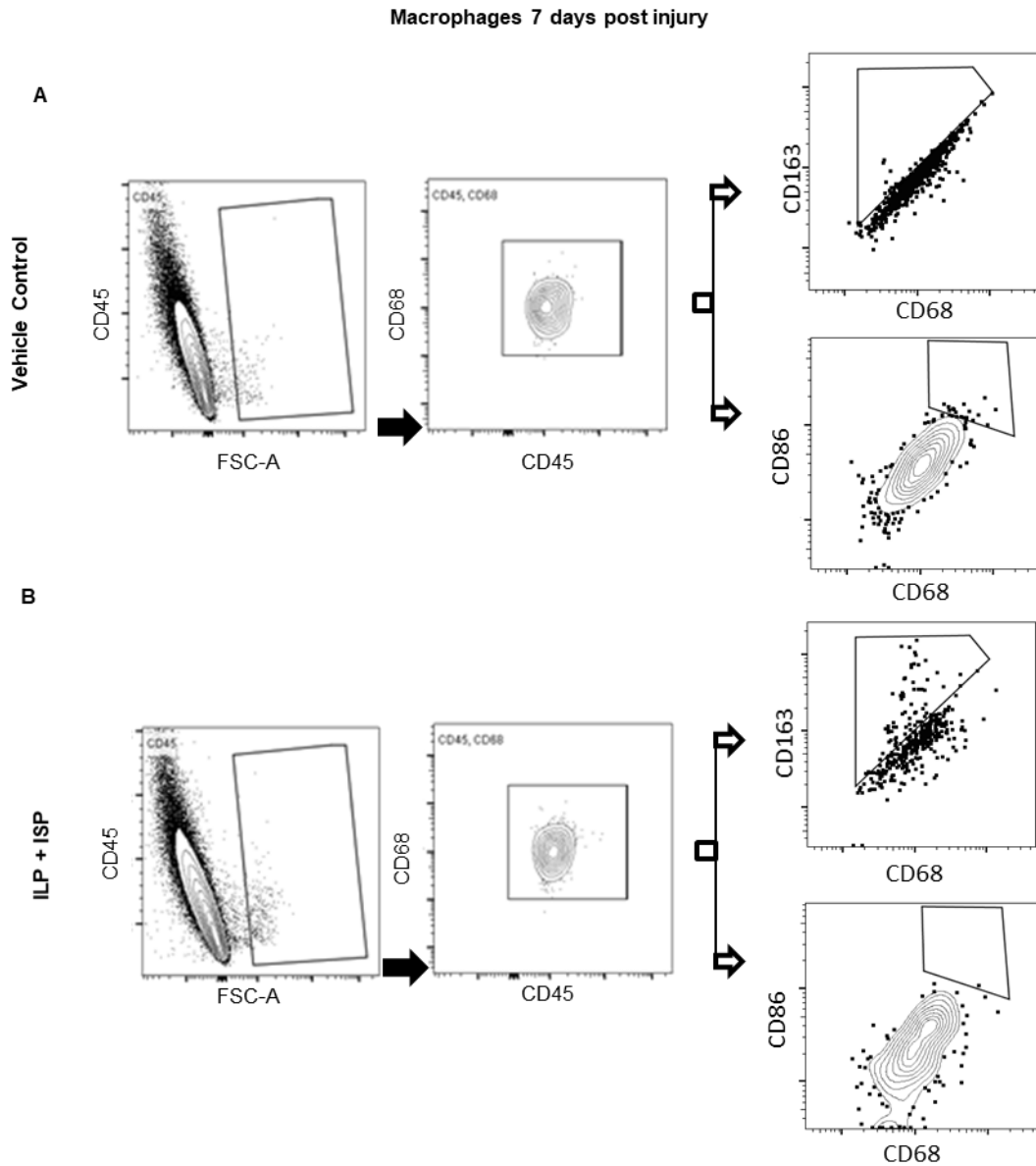
(A-B) For flow cytometric analysis of infiltrating macrophages, a total of 200,000 events were captured. Singlets were separated using FSC-H versus FSC-A, and CD45^{+}/CD68+ cells were identified for subsequent phenotypical analysis. To identify different phenotypes of macrophages, cells were gated for CD86 as M1 or CD163 as M2 macrophages as shown in A-B.

Supplementary Figure 4.4: Summary of flow cytometry gating strategy used for phenotypical analysis of helper T cells in injured spinal cord tissue.
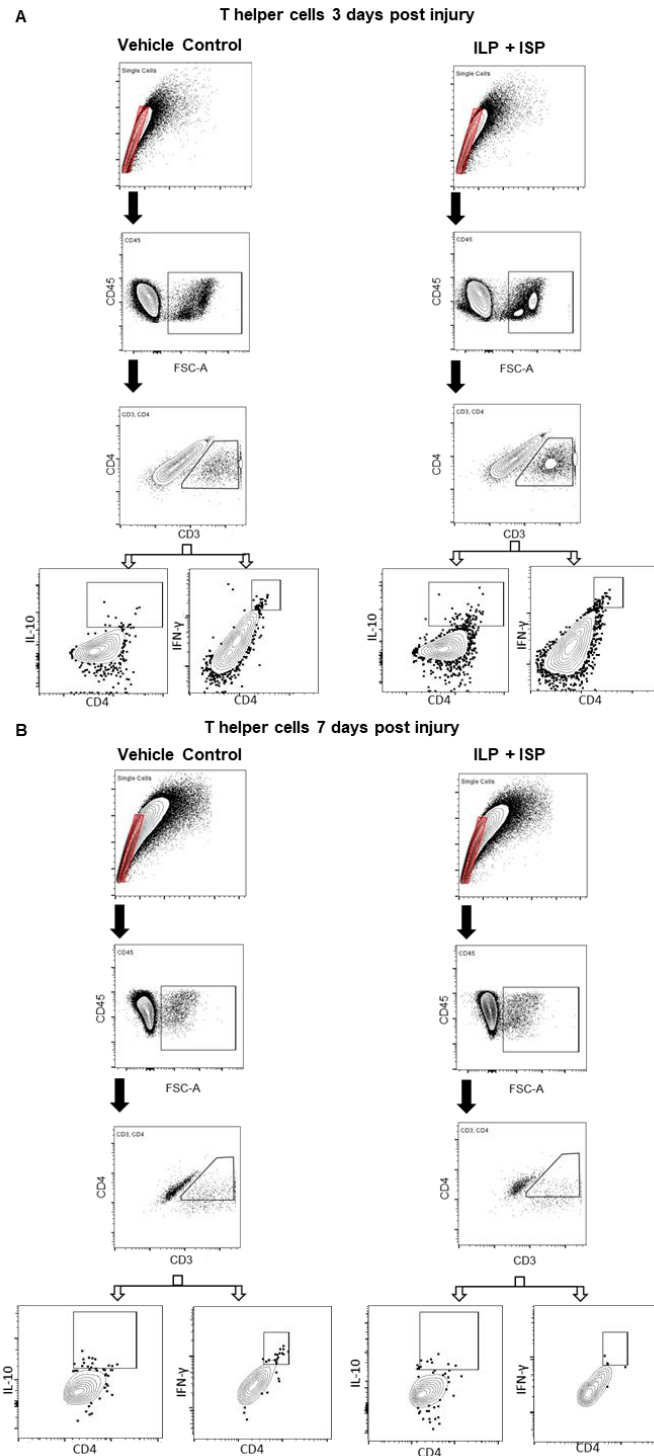
Supplementary Figure 4.4: Summary of flow cytometry gating strategy used for phenotypical analysis of helper T cells in injured spinal cord tissue.

(A-B) Flow cytometric analysis of infiltrating T cells started with identifying singlets using FSC-H versus FSC-A, and CD45+/CD3+/CD4+ cells as helper T cells. Next, to identify regulatory versus effector T cell phenotypes, cells were gated for CD4/IL-10 as T_{reg} or CD4/IFNγ as effector T cells.

Supplementary Figure 4.5: Microglia express LAR and PTPσ.
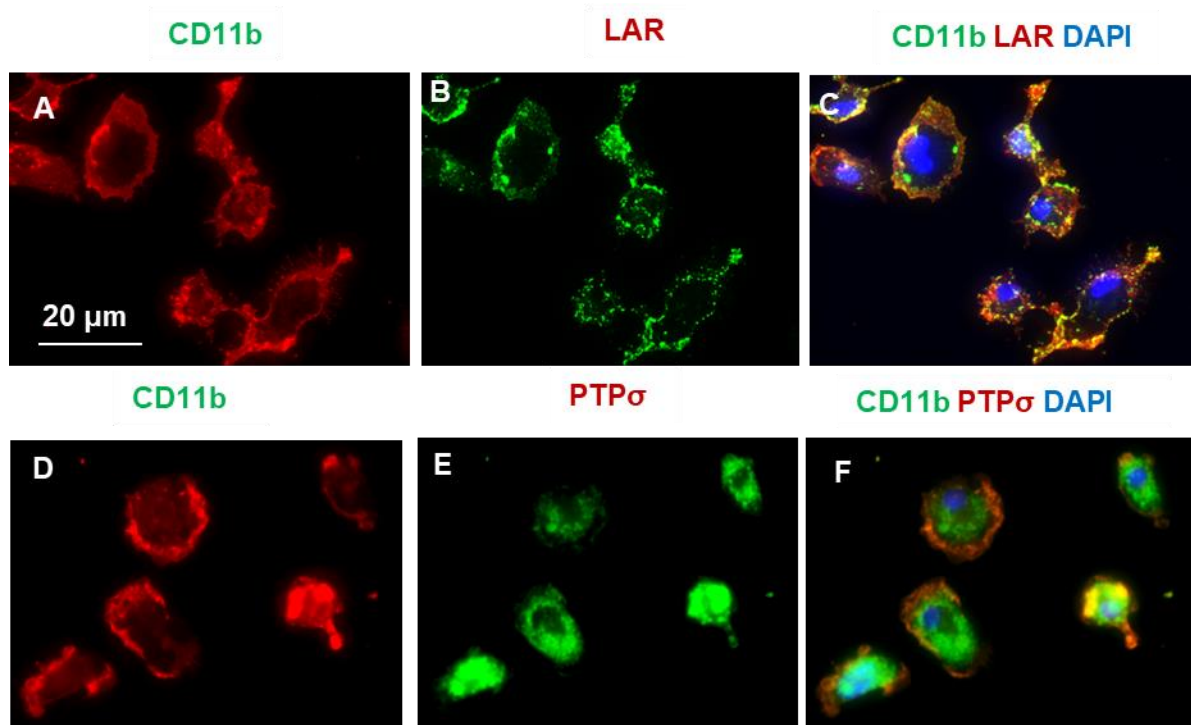
(A-F) Immunocytochemistry on cultures of primary microglia confirms expression of LAR (A-C) and PTPσ (D-F) in microglia marked by CD11b.

Chapter 5: Discussion

5.1 General Overview of Findings

This thesis focused on uncovering the role and mechanisms of CSPGs and their signaling receptors LAR and PTPσ in modulating the endogenous cell response and neuroinflammation processes following SCI. First, using direct *in vitro* assays which mimic the matrix of the SCI-niche, we demonstrate that CSPGs negatively regulate several properties of spinal cord derived adult NPCs including their growth, integration, survival, proliferation, and oligodendrocyte differentiation (Dyck et al. 2015). Next, using genetic (Dyck et al. 2015) and pharmacological approaches (Dyck et al, two manuscripts under review) we identified, for the first time, that CSPGs regulate NPCs by signaling through both LAR and PTPσ receptors. Mechanistically, we show that activation of the Rho/ROCK pathway seems to be a central mechanism by which CSPGs regulate the properties of NPC since Y-27632, a ROCK inhibitor, was able to block nearly all of the inhibitory effects of CSPGs on NPCs (Dyck et al. 2015). At the intracellular level, activation of CSPGs signaling decreased the phosphorylated state of Akt and Erk1/2 in NPCs, which appear to be downstream effectors of LAR and PTPσ signaling in spinal cord NPCs (Dyck et al. 2015). We extended these studies to OPCs demonstrating that CSPGs negatively modulate their attachment, survival and maturation *in vitro* by signaling through LAR and PTPσ and activation of the Rho/ROCK pathway. These novel *in vitro* findings uncovered the plausible cellular mechanisms by which upregulation of CSPGs within the microenvironment of injury influence the regenerative response of endogenous precursor cells, and identified potential targeted interventions for efficient optimization of cell-based repair strategies for SCI.

In rat compressive SCI, we next demonstrated that LAR and PTPσ play critical roles in regulating the endogenous cell response to injury. Perturbing LAR and PTPσ attenuates

oligodendrocyte apoptosis while increasing endogenous oligodendrogenesis. Interestingly, we found a critical role for LAR and PTPσ receptors in modulating the immune response in SCI. Blockage of LAR and PTPσ allowed a shift from an M1 pro-inflammatory to an M2 pro-regenerative response in microglia that promoted oligodendrogenesis in NPCs. M2 derived IL-10 expression was shown to be an underling mechanism for the increased oligodendrocyte differentiation. We also found, in SCI and microglia cultures, that the modulatory effects of LAR and PTPσ signaling is mediated through RhoA activation and can be attenuated by ILP/ISP therapy. Moreover, our *in vitro* studies identified that CSPGs limit the ability of microglia for mobilization and phagocytosis; characteristics of a reparative phenotype in microglia (David and Kroner 2011). Altogether, our findings have identified novel inhibitory roles for CSPGs and their LAR and PTPσ receptors in modulating repair processes in the injured spinal cord and new underlying mechanisms of functional recovery that are consistently observed when CSPGs are targeted by various strategies after SCI (Karimi-Abdolrezaee et al. 2010; Bradbury et al. 2002a; Lang et al. 2015; Fisher et al. 2011).

5.2 General Discussion

CSPGs are markedly upregulated following SCI (Buss et al. 2009) and have long been implicated in limiting the endogenous repair mechanisms (Dyck and Karimi-Abdolrezaee 2015). ChABC treatment, which degrades CSPGs (Bradbury et al. 2002a), has resulted in varying degrees of functional and physiological improvements after injury (Barritt et al. 2006a; Bradbury et al. 2002a; Cafferty et al. 2008; Caggiano et al. 2005; Carter, McMahon, and Bradbury 2011; Carter et al. 2008; Chau et al. 2003; Fouad et al. 2009; Fouad et al. 2005; Galtrey et al. 2007; García-Alías et al. 2011; Houle et al. 2006; Ikegami, Nakamura, Yamane, Katoh, Okada, Iwanami, Watanabe,

Ishii, Kato, Fujita, Takahashi, Okano, et al. 2005; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Massey et al. 2008; Massey et al. 2006; Moon et al. 2001; Tester and Howland 2008; Tom, Kadakia, et al. 2009; Tom, Sandrow-Feinberg, et al. 2009; Wang, Ichiyama, et al. 2011; Yick et al. 2007). In the past decade, a plethora of data has demonstrated that CSPGs negatively modulate several important aspects of spinal cord repair including axonal sprouting, regeneration and conduction as well as replacement of oligodendrocytes, and as a result, remyelination (Chen et al. 2002; Barritt et al. 2006a; Massey et al. 2006; Alilain et al. 2011; Galtrey and Fawcett 2007; Bradbury et al. 2002a; Fournier, Takizawa, and Strittmatter 2003; Cafferty et al. 2007; Massey et al. 2008; Tom, Sandrow-Feinberg, et al. 2009; Petrosyan et al. 2013; Arvantan et al. 2009; Hunanyan et al. 2010; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2012; Pendleton et al. 2013; Lau et al. 2012a; Larsen et al. 2003a). In recent years, the identification of specific CSPGs receptors PTPσ (Shen et al. 2009a; Fry, Chagnon, Lopez-Vales, et al. 2010) and LAR (Fisher et al. 2011) was a breakthrough in the field, and allowed for new insights into CSPGs mechanism in CNS regeneration and repair. This thesis elucidates the functional role of LAR and PTPσ signaling in SCI using a relevant preclinical model and various primary cell types *in vitro* including NPCs, OPCs and microglia.

5.3 CSPGs Signaling Receptors: LAR and PTPσ

LAR and PTPσ are widely expressed in adult CNS neurons and are shown to mediate CSPGs inhibitory effects on axonal regeneration *in vitro* and in SCI (Fisher et al. 2011; Shen et al. 2009a). Expression of PTPσ in neurons has been associated with failure in axonal growth in lamprey and rodents (Zhang et al. 2014; Shen et al. 2009a; Fry, Chagnon, Lopez-Vales, et al. 2010). PTPσ knockout mice show better CST regeneration following SCI (Shen et al. 2009a; Fry, Chagnon,

Lopez-Vales, et al. 2010) and enhanced axonal regeneration after optic nerve and peripheral nerve injuries (McLean et al. 2002; Thompson et al. 2003; Sapeiha et al. 2005). In lamprey, reticulospinal neurons which fail to regenerate following a spinal cord transection were shown to highly express PTPσ, whereas those which regenerated past the glial scar had reduced expression of PTPσ (Zhang et al. 2014). Similarly, LAR has been implicated in limiting axonal regeneration and functional outcomes following injury (Fisher et al. 2011; Xu et al. 2015). LAR knockout mice have shown improved locomotor function, CST regeneration, and serotonergic axonal sprouting following SCI (Fisher et al. 2011; Xu et al. 2015).

Here, our new evidence extends these concepts by revealing that spinal cord NPCs can be influenced by SCI-induced upregulation of CSPGs due to their expression of both LAR and PTPσ receptors (Dyck et al. 2015). Using siRNA gene silencing, as well as PTPσ knockout mice studies, we demonstrate for the first time that downregulation of LAR or PTPσ partially reversed CSPGs inhibitory effects on NPCs with additive effects after their co-inhibition (Dyck et al. 2015). Parallel studies by other groups have also identified the important role PTPσ plays in mediating CSPG effects on OPCs (Pendleton et al. 2013). Collectively, these studies have identified that modulation of LAR and PTPσ receptors is a viable approach to target CSPG effects in SCI. These findings have led to the development of novel synthetic peptides designed to block LAR and PTPσ receptors including ILP and ISP (Lang et al. 2015; Xu, Zhu, and Heinemann 2006). These pharmacological approaches have allowed studying the impact of CSPGs signaling in clinically relevant models of SCI.

ILP and ISP are two novel intracellular peptides designed to block LAR (Xu, Zhu, and Heinemann 2006), and PTPσ signaling (Lang et al. 2015), respectively. These peptides have been tested both *in vitro* and *in vivo* to target CSPGs signaling in neurons (Lang et al. 2015; Fisher et

al. 2011). Blocking LAR with ILP resulted in increased axonal growth of serotonergic fibers which was accompanied by improvements in functional recovery (Fisher et al. 2011). Similarly, inhibition of PTPσ with ISP can significantly improve both bladder and locomotor functions after contusive SCI in rats (Lang et al. 2015). Targeting PTPσ promoted remarkable serotonergic re-innervation below the level of SCI that contributed to functional recovery (Lang et al. 2015). Interestingly, pharmacological blockade of serotonergic receptors was not sufficient to entirely reverse functional improvement of ISP treatment suggesting additional contributing mechanisms. These observations substantiate our findings here that LAR and PTPσ have additional roles including regulation of cell differentiation and immune response in the injured spinal cord.

5.4 CSPGs regulation of endogenous cell response in SCI

Utilizing ILP and ISP, we aimed to address the unexplored question of how CSPGs impact the behaviour of spinal cord precursor cells and their ability to differentiate into oligodendrocytes in SCI. In agreement with our genetic knockdown studies in Chapter 1 (Dyck et al. 2015), we found that inhibition of each receptor individually by ILP or ISP was effective to partially reverse CSPG effects on NPCs in culture. However, the co-inhibition of LAR and PTPσ with ILP and ISP maximized their individual beneficial effects. These findings were confirmed in cultures of primary OPCs where we demonstrated an important role of both LAR and PTPσ in mediating CSPGs inhibitory effects on attachment, cell spreading, survival and maturation of OPCs. While solitary treatment with ILP or ISP was able to significantly limit CSPGs detrimental effects on OPCs, co-inhibition of ILP and ISP resulted in more significant effects. Previous reports also demonstrated that CSPGs limit OPCs process outgrowth and myelination *in vitro* (Pendleton et al.

2013; Lau et al. 2012a). Given our *in vitro* findings in both NPCs and OPCs, we therefore delivered ILP and ISP in combination following SCI.

In rat SCI, for the first time, we demonstrate that co-inhibition of LAR and PTPσ with ILP/ISP remarkably enhances oligodendrogenesis in the subependymal as well as in the white matter regions of the spinal cord where adult NPCs and OPCs reside, respectively. These beneficial effects were also accompanied by a reduction in the number of newly generated astrocytes. This is particularly important as it is known that SCI niche instructs NPCs to give rise to astrocytes with only a limited number differentiating into oligodendrocytes (Barnabe-Heider et al. 2010; Meletis et al. 2008; Sabelström et al. 2013; Karimi-Abdolrezaee et al. 2010; Mothe and Tator 2005a). Previous studies from our group in SCI also showed that ChABC treatment can increase endogenous oligodendrogenesis (Karimi-Abdolrezaee et al. 2012) and promoted oligodendrocyte differentiation of transplanted NPCs (Karimi-Abdolrezaee et al. 2010). It is important to note that detection of newly generated oligodendrocytes by BrdU incorporation approaches does not allow us to discern whether oligodendrogenesis occurred through NPCs or OPCs. Therefore, the robust increase in endogenously generated oligodendrocytes following ILP and ISP treatment, or in our previous ChABC studies in rat SCI, is likely attributed to modulation of both NPC and OPC populations. Taken together, our new findings show that over-activation of CSPGs and LAR/PTPσ signaling seems to be an underlying cause of inadequate oligodendrogenesis following SCI. Replacement of oligodendrocytes and remyelination contribute to recovery of function after SCI (Karimi-Abdolrezaee et al. 2012; Karimi-Abdolrezaee et al. 2010; Hawryluk et al. 2014; Alizadeh, Dyck, and Karimi-Abdolrezaee 2015). Therefore, targeting LAR and PTPσ represents a potential viable repair strategy for promoting oligodendrocyte replacement following SCI.

5.5 CSPGs and oligodendrocyte cell death in SCI

In addition to active inhibition of oligodendrogenesis, we have provided novel evidence that CSPGs can induce caspase 3 mediated apoptosis in both NPCs and OPCs *in vitro*, and importantly in mature oligodendrocytes in rat SCI, which was overcome by blocking LAR and PTPσ receptors. Oligodendrocytes are highly susceptible to cell death and undergo pronounced apoptosis in a delayed fashion after SCI (McTigue, Wei, and Stokes 2001). Since mature oligodendrocytes are terminally differentiated, apoptosis depletes their population leading to progressive demyelination. Thereby, it is critical to protect oligodendrocytes following SCI.

We found that ILP/ISP therapy can attenuate overall activation of caspase 3 at 3 and 7 days following SCI. Importantly, at 7 days post injury, ILP/ISP treated rats had a significantly decreased number of cleaved caspase 3 positive mature oligodendrocytes around the lesion site. Of note, these acute/subacute time-points coincide with the peak of oligodendrocyte apoptosis in our rat compressive SCI model (Robins-Steele, Nguyen, and Fehlings 2012). We also observed a significant increase in MBP immunodensity at 28 days post injury in ILP/ISP treated animals reflecting reduced oligodendrocyte cell loss and/or increased oligodendrocyte replacement that we observed following ILP/ISP treatment. A few studies have implicated CSPGs in modulating neuronal atrophy or death in SCI (Carter, McMahon, and Bradbury 2011; Carter et al. 2008), however to our knowledge; this is the first study that links CSPGs to oligodendrocyte apoptosis. ChABC treatment has previously been shown to limit neuronal atrophy after SCI (Carter, McMahon, and Bradbury 2011; Carter et al. 2008). Intrinsically labeled corticospinal neurons in layer V of the sensorimotor cortex undergo progressive atrophy, with shrinkage of cell bodies but no evidence of cell death at four weeks post injury in a dorsal column injury model of mouse SCI (Carter et al. 2008). Cellular atrophy was overcome by ChABC treatment (Carter et al. 2008) and

was later shown to also occur in rubrospinal neurons in a C5 lateral column SCI model (Carter, McMahon, and Bradbury 2011). Interestingly, in a transection model of SCI in lamprey, high expression of PTPσ in neurons was directly correlated with higher expression of caspase activation indicating a role for PTPσ in the retrograde neuronal death following SCI (Zhang et al. 2014). The same group later demonstrated that RhoA activation was also associated with apoptosis and degeneration in the same transection model of SCI in lamprey (Hu and Selzer 2017), which is in agreement with our observation in rat compressive SCI here. Altogether, our findings have provided novel evidence that CSPGs contribute to apoptosis in oligodendrocytes, and pose a challenge on their replacement by limiting endogenous oligodendrogenesis in SCI.

5.6 Intracellular mechanisms of CSPGs: Rho/ROCK, Akt and Erk1/2

In this study, we have identified several key downstream intracellular pathways that are modulated by CSPGs signaling. We found that RhoA plays a central role in mediating the inhibitory effects of CSPGs through LAR and PTPσ. It is known that SCI results in activation of RhoA that is an underlying cause of regeneration failure in axons (Eftekharpour et al. 2016; McKerracher and Anderson 2013; McKerracher, Ferraro, and Fournier 2012). Blocking the Rho/ROCK pathway is known to promote axonal regeneration and functional recovery following SCI (Monnier et al. 2003; Dergham et al. 2002; Fournier, Kalb, and Strittmatter 2000; Ramer, Borisoff, and Ramer 2004). Our new findings indicate that LAR and PTPσ regulate RhoA activation after SCI, and their inhibition with ILP/ISP was able to significantly attenuate the SCI induced increase in RhoA activity at 3 days post-SCI. Interestingly, these data also established a temporal correlation between the increase in RhoA activity and induced levels of cleaved caspase 3 after SCI as both RhoA activity and caspase 3 activation were attenuated by ILP/ISP treatment. Our *in vitro* studies

also identified the involvement of RhoA/ROCK pathway in CSPGs induced activation of caspase 3 in NPCs and OPCs, which was also mediated by LAR. Rho activation has also been associated with p75 mediated apoptosis in neurons and glial cells following SCI (Dubreuil, Winton, and McKerracher 2003), however its link to LAR and PTPσ has not be reported. RhoA activation by LAR and PTPσ signaling has been shown in neuronal cultures by other groups using genetic manipulations of LAR (Fisher et al. 2011; Ohtake et al. 2016) and PTPσ (Ohtake et al. 2016). Ohtake and colleagues demonstrated that activation of LAR and PTPσ each individually can result in the activation of RhoA in cerebellar granular neurons (Ohtake et al. 2016). Here, our *in vitro* work also showed the negative impact of CSPGs on cleaved caspase 3 mediated apoptosis in NPCs and OPCs in which was restored following inhibition of the Rho/ROCK pathway with Y-27632 (Dyck et al. 2015). Moreover, our *in vitro* findings in microglia further identified the Rho/ROCK pathway as a downstream mediator in CSPGs and LAR/PTPσ signaling in microglia regulation. Altogether, our new findings and that of others, have identified an association between CSPGs signaling and the RhoA/ROCK pathway that seems to serve as a plausible mechanism of cell death in neurons, oligodendrocytes and NPCs. However, this does not exclude the involvement of other RhoA independent mechanisms for the reduced oligodendrocyte cell death we observed following ILP/ISP treatment after SCI. While further work is required to elucidate the mechanisms behind these correlations, we propose that other confounding factors such as the ability of ILP and ISP to modulate cytokine release may have also contributed to the reduced oligodendrocyte apoptosis after SCI. Our microglia and SCI studies provided evidence that ILP/ISP treatment moderates the release of pro-inflammatory cytokines TNF-α and IL-1β, which are known to induce cell death (Ferguson et al. 2008; Song et al. 2006; Miller et al. 2005; Sato et al. 2012; Takahashi et al. 2003).

Our findings also unraveled that exposure to CSPGs inhibits phosphorylation of both Akt and Erk1/2 in spinal cord NPCs, which is mediated by both LAR and PTPσ receptors (Dyck et al. 2015). Subsequent genetic studies in neurons also identified that activation of both LAR and PTPσ results in dephosphorylation of Akt and Erk1/2 (Ohtake et al. 2016). We demonstrate that inhibiting LAR and PTPσ by genetic knockdown or by ILP and ISP promotes Akt and Erk1/2 phosphorylation in NPCs on CSPGs substrate. PI3K/Akt and MAPK/Erk signaling have previously been shown to promote NPCs proliferation (Chan et al. 2013) and enhance oligodendrocytes survival and differentiation *in vitro* and *in vivo* (Flores et al. 2000; Rafalski et al. 2013; Rowe et al. 2012). Downstream effector of the PI3K/Akt, mTOR, is critical for oligodendrocyte differentiation (Tyler et al. 2009) and myelination (Flores et al. 2008; Goebbels et al. 2010). MAPK/Erk signaling also plays a central role in oligodendrocyte process extension and myelination (Ashii et al. 2012; Furusho et al. 2012a; Fyffe-Maricich et al. 2011b; Flores et al. 2008). Our knockdown studies identified that PTPσ receptor appears to be the key mediator of Erk1/2 dephosphorylation in NPCs. Collectively, our findings provide novel evidence that CSPGs signaling negatively regulates activation and oligodendrocyte differentiation of spinal cord NPCs by repressing Akt and Erk1/2 activities.

5.7 CSPGs modulate neuroinflammation following SCI

Neuroinflammation is a complex process, which was originally considered by many in the SCI field to be ultimately detrimental. More recent evidence has shown that modulating the inflammatory response to an M2 phenotype promotes functional outcomes in multiple neurological conditions including MS and SCI (Miron and Franklin 2014). The first phase of neuroinflammation involves neutrophils who are the first cells to enter the spinal cord after insult

(Neirinckx et al. 2014). Neutrophils are recruited to the injury site within 6 hours after injury and their numbers peak within 24 hours (Neirinckx et al. 2014). They are generally thought to exacerbate the injury process by damaging neurons, glial cells and endothelial cells, releasing toxic ROS and proteases (Dumont et al. 2001). Here, we demonstrate that ILP and ISP treatment limits the overall acute infiltration of neutrophils evidenced by their MPO activity following SCI. Reducing neutrophil infiltration is associated with reduced tissue damage and as a result improvement of functional outcomes after SCI (Neirinckx et al. 2014; Dumont et al. 2001). We also propose that the ability of ISP and ILP to attenuate neutrophil activities may contribute to functional recovery which we and other observed in previous studies (Fisher et al. 2011; Lang et al. 2015).

The second phase of inflammation involves the recruitment of macrophages to the injury site (David and Kroner 2011; Miron and Franklin 2014). Our flow cytometry and molecular analyses indicate that ILP and ISP treatment has no effects on the overall number of microglia/macrophage in the spinal cord lesion, and they rather modulate their phenotype. This observation is indeed promising as macrophage infiltration is necessary following injury. Evidence shows that ablating the macrophage cellular pool is associated with worse outcomes after SCI (Shechter et al. 2009). Notably, we found that inhibition of LAR and PTPσ promoted an M2 phenotype in macrophages (CD45+/CD68+/CD163+) after SCI characterized by an increase in IL-10 and arginase-1 protein expression. Our findings are in agreement with recent studies where ChABC treatment induced an anti-inflammatory response marked by upregulation in both arginase-1 and IL-10 expression after SCI (Bartus et al. 2014; Didangelos et al. 2014). In those studies, the anti-inflammatory M2 response was associated with reduced cavitation after SCI (Bartus et al. 2014; Didangelos et al. 2014). Of note, the impact of microglia/macrophages on the

repair process is determined by their activation state, either being classically activated M1, or alternatively activated M2 (David and Kroner 2011; Kigerl et al. 2009). Initially, there are relatively equal numbers of M1 and M2 microglia/macrophages in the SCI lesion; however, over the time, this landscape switches predominantly towards an M1 phenotype which is associated with cell death and tissue degeneration through their expression of ROS, RNS, proteases and pro-inflammatory cytokines (Kigerl et al. 2009; Takahashi et al. 2003; David and Kroner 2011). Conversely, alternatively activated M2 macrophages are responsible for phagocytosis of myelin debris and secretion of factors that promote axonal growth and oligodendrocyte differentiation (Miron and Franklin 2014). Inducing an M2 phenotype is generally associated with improved outcomes after CNS injury (Miron et al. 2013). Transplantation of exogenous M2 cells promotes functional recovery following SCI indicating their ability to foster a regenerative program in the injured spinal cord (Rapalino et al. 1998).

5.8 Role of M2 microglia in oligodendrogenesis of NPCs

The importance of crosstalk between NPCs and inflammatory cells is becoming increasingly more appreciated in regulating cell replacement in CNS injury (Kokaia et al. 2012). For example, pro-inflammatory cytokines such as TNFα and IL-6 negatively affect hippocampal neurogenesis in the LPS-treated brain whereas anti-inflammatory cytokines such as insulin-like growth factor-1 (IGF-1) promote healing and cell renewal (Butovsky et al. 2006; Monje, TOda, and Palmer 2003). Our complimentary *in vitro* assessment demonstrated that M2 microglia promote NPC proliferation and oligodendrocyte differentiation. Although multiple trophic factors likely contributed to these effects, IL-10 was critically important for promoting oligodendrocyte differentiation of NPCs. Similarly, Miron et al recently demonstrated that condition media of M2 promotes OPC maturation

and differentiation *in vitro* (Miron et al. 2013). Thereby, the increase in endogenous proliferation and oligodendrocyte differentiation that we have observed following ILP/ISP therapy in rat SCI may be attributed to changes in M1:M2 ratio of inflammatory cells after blockade of LAR and PTPσ signaling. These new findings are important to increase our understanding of the significance of inflammation following insult to the CNS, and indicate the importance of the promoting growth factors and cytokines associated with an M2 inflammatory response. Given that IL-10 appears to be a major contributor to the increased oligodendrogenesis that we observed following ILP and ISP treatment, we next uncovered which cell types contributed to its upregulation. In SCI, IL-10 expressing M2 macrophages reside in the spinal cord around the lesion penumbra whereas M1 microglia typically reside within the lesion epicenter (Shechter et al. 2009). We observed significantly more IL-10 expressing OX42+ microglia/macrophages in the penumbra of the spinal cord lesion with ILP and ISP treatment indicating that inhibiting LAR and PTPσ signaling promotes M2 polarization in these cells.

To provide additional mechanistic insights into the modulatory role of CSPGs and LAR and PTPσ signaling, we utilized a primary *in vitro* model of microglia. Our studies in primary microglia revealed that while CSPGs do not seem to be an inducer of M1 phenotype in microglia *per se*; their presence in the milieu of M1 microglia promotes and/or maintains their pro-inflammatory phenotype. We demonstrate that CSPGs promote production of M1 markers IL-1β and nitrite in microglia. Interestingly, while CSPGs-induced increase in IL-1β was attenuated by ILP/ISP, nitrite production was not mediated by LAR and PTPσ signaling. This evidence suggests that CSPGs seem to regulate nitrite production in M1 microglia through other mechanisms, which needs further elucidation. Interestingly, presence of CSPGs also supressed IL-10 release by M2 polarized microglia, which was ameliorated by inhibition of LAR and PTPσ signaling. These *in*

vitro findings supported our SCI assessments uncovering a novel regulatory role for LAR and PTPσ in modulating microglia polarization that had not been identified previously.

Our microglia *in vitro* assessment also identified an inhibitory role for CSPGs in regulating microglia mobilization, which was overcome by ILP/ISP treatment and ChABC degradation of CSPGs. Migration of activated microglia to the site of spinal cord and brain injury is important for their contribution to the repair process including phagocytosis of debris and wound healing (Davalos et al. 2005; David and Kroner 2011). *In vivo* imaging of microglia has identified them as highly motile cells in their environment (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). Our data suggest that presence of CSPGs limit microglia mobility, and LAR and PTPσ appear to partially mediate this effect as ILP/ISP treatment was able to significantly attenuate the negative effects of CSPGs on microglia mobilization. Importantly, mobilization of microglia is essential for their wound healing functions such as phagocytosis. In this regard, our direct *in vitro* systems also revealed that M1 polarization or the presence of CSPGs restricted microglia phagocytosis. We also found that the effects of CSPGs on microglia phagocytosis seem to be mediated through LAR/PTPσ signaling as well as through activation of the Rho/ROCK pathway. Interestingly, in M1 microglia, inhibition of LAR/PTPσ also promoted phagocytosis in the absence of CSPGs suggesting that these receptors may interact with other ligands or have other functions. Nevertheless, promoting the ability of microglia for phagocytosis is beneficial for the repair process (David and Kroner 2011). It is known that impaired phagocytosis by microglia is correlated with limited tissue regeneration in traumatic CNS injuries and neurodegenerative diseases (Neumann, Kotter, and Franklin 2009; David and Kroner 2011; Kroner et al. 2014). Our findings provide evidence suggesting that long-lasting upregulation of CSPGs in the ECM of SCI may underlie the ineffective and prolonged clearance of debris in the injured spinal cord (Kroner

et al. 2014). To our knowledge, our study is the first to unravel the impact of CSPGs, and LAR and PTPσ signaling on microglia mobilization and phagocytosis.

CSPGs also appeared to modulate nitrite production *in vitro.* However, while CSPGs-induced increase in IL-1β was attenuated by ILP/ISP, nitrite production was not mediated by LAR and PTPσ signaling. This evidence suggests that CSPGs seem to regulate nitrite production in M1 microglia through other mechanisms, which needs further elucidation. Our findings indicate a role for LAR and PTPσ in regulating T cell response after SCI. While ILP/ISP had no effects on the overall number of T cells, there was a significant increase in the number of T_{regs} and a decrease in the number of T effector cells. We also observed an increase in the protein expression of FOXP3, a marker for T_{regs}. Interestingly, our complementary immunostaining confirmed that ILP/ISP treatment promotes CD3IL-10 expressing T-cells around the SCI lesion. Of note, in the subacute/chronic stages of injury, macrophages recruit FOXP3 positive T_{regs} cells, which are associated with tissue repair and functional recovery in mice (Raposo et al. 2014). While our initial findings suggest that targeting LAR and PTPσ can promote T_{regs} in SCI, further *in vitro* and *in vivo* studies are required to identify CSPGs mediated mechanisms in T cells regulation.

Taken together, we have uncovered that targeting LAR and PTPσ signaling can harness the beneficial role of inflammatory cells following SCI. We provide parallel *in vivo* and *in vitro* evidence that positive modulation of the neuroinflammatory response by ILP/ISP appears to contribute to the improved oligodendrocyte replacement and preservation that we have observed in this study. Our findings have also identified new underlying mechanisms by which targeting LAR and PTPσ enhanced functional recovery in previous studies from our group and others in rat SCI (Fisher et al. 2011; Lang et al. 2015).

5.9 Discussion of other plausible mechanisms

Although LAR and PTPσ receptors play an important role in CSPGs signaling cascade (Coles et al. 2011; Shen et al. 2009a; Ohtake et al. 2016; Fry, Chagnon, Lopez-Vales, et al. 2010), CSPGs are also shown to bind to Nogo66 receptors, NgR1 and NgR3 (Dickendesher et al. 2012). Nogo66 receptors were originally identified for their role in mediating the inhibitory effects of myelin associated inhibitor Nogo on axon regeneration in the CNS (Fournier, GrandPre, and Strittmatter 2001; Mehta et al. 2007). However, studies by Dickendesher and colleagues identified that NgR1 and NgR3 also show high affinity binding to CSPGs (Dickendesher et al. 2012). Accordingly, with inhibition of LAR and PTPσ with ILP/ISP, CSPGs may still exert their effects by signaling through other mechanisms including NgR1 and NgR3. Moreover, PTPσ and LAR have shown the affinity to interact with other ligands such as heparan sulfate proteoglycan (HSPGs) (Dyck and Karimi-Abdolrezaee 2015; Fox and Zinn 2005; Aricescu et al. 2002). Interestingly, interaction between HSPGs-PTPσ and CSPGs-PTPσ has different outcomes. HSPGs-PTPσ activation promotes axon growth and synapse formation (Coles et al. 2011; Aricescu et al. 2002), whereas CSPG-PTPσ signaling inhibits regeneration (Coles et al. 2011; Shen et al. 2009a). Similarly, while LAR appears to binds to CSPGs with high affinity (Fisher et al. 2011), it also interacts with HSPGs (Fox and Zinn 2005) and homophillically to itself (Yang et al. 2003; Yang et al. 2005). Thus, ILP/ISP therapy in our studies may have influenced interaction of LAR and PTPσ with other ligands.

5.10 Study Limitations

The data presented in this thesis identify a novel and crucial role for CSPGs-LAR/PTPσ axis in NPCs regulation and repair processes in SCI. However, like any other work, there are some limitations that should be addressed to interpret the results and provide directions for future studies.

One limitation in this study was our cell differentiation assessments in our rat SCI model. While our BrdU assessments convincingly determined endogenous oligodendrogenesis in the spinal cord, this methodology would not allow attributing the new oligodendrocytes to NPCs to OPCs. Of note, BrdU labelling tracks all cell types undergoing cell division after injury including NPCs and OPCs. To differentiate the role of each cell type in response to ILP/ISP treatment, genetic models were essential where ependymal cells (NPCs) and OPCs are labelled within the spinal cord similar to the approach that Barnabe-Heider and colleagues employed using FoxJ1-CreER and Olig2-CreER transgenic mice (Barnabe-Heider et al. 2010). Given this limitation of our rat SCI model, we designed parallel *in vitro* studies to investigate the effects of ISP/ILP on the behaviour of both NPCs and OPCs in direct assays. However, it is noteworthy to mention that while transgenic models are invaluable tools in unraveling mechanisms, they are not disease relevant. The rat model of SCI that we have employed more closely represents the pathophysiology of human SCI (Jakeman et al. 2000) and it has been recognized as a clinically relevant experimental model for developing therapies for SCI. Thereby, while we have not been able to specifically study the NPCs/OPCs population in SCI, we have been able to identify the role of CSPGs and LAR and PTP receptors in overall oligodendrocyte replacement in a clinically relevant model of SCI.

Another limitation in our study was the extent of our flow cytometry analysis. While there in an extended antibody panel available for flow cytometry in mouse tissue, the number of effective antibodies for rat tissue is rather limited especially for intracellular markers. Accordingly, we were limited with the composition of our antibodies. However, we were able to include major immune phenotypes in our study and also used complementary Western blotting to study key inflammatory cytokines and mediators in our studies.

5.10 Future Directions

Findings from my thesis have identified novel mechanisms through which CSPGs mediate their inhibitory effects on oligodendrocyte replacement following SCI providing new approaches to target CSPGs in cell therapies. Administration of ILP and ISP in rat SCI led to a robust increase in oligodendrocyte preservation and replacement. Interestingly, our *in vitro* and *in vivo* assessments indicate that these effects are mediated, at least partially, by inducing an anti-inflammatory and pro-regenerative immune response following SCI. These findings suggest that targeting CSPGs in SCI can foster a permissive microenvironment for NPCs and potentiate their regenerative response. Capitalizing on these new findings, we further hypothesize that combining ILP and ISP treatment with NPCs transplantation will optimize the integration, survival, and oligodendrocyte differentiation of engrafted NPCs and maximize functional recovery in SCI. As a future direction, we aim to determine the efficacy of ILP and ISP treatment in promoting the outcomes of NPCs transplantation in rat model of SCI. We will also evaluate the effects of ILP/ISP on remyelination using our transplantation model. While we will also assess the effects of ILP/ISP on endogenous remyelination, NPC transplantation can provide a more effective system for this assessment. We will evaluate the potential benefits of combining ILP/ISP co-treatment and NPC transplantation in improving functional recovery in rat SCI. We anticipate that inhibiting CSPGs signaling with ILP and ISP treatment will optimize the therapeutic benefits of transplanting NPCs for SCI repair in an additive fashion and will result in a more meaningful functional recovery after SCI. These future studies are crucial for optimizing the outcomes of current NPC based-cell therapies for SCI. Upregulation of CSPGs in the matrix of glial scar is an inevitable consequence

of SCI. Hence, developing clinically-relevant strategies to target CSPGs notorious effects is a vital step in advancing the promise of NPCs therapies from preclinical testing to SCI patients.

5.11 Clinical Implications

SCI is a devastating disease that is estimated to affect 2.5 million people world-wide (Fehlings, Wilson, and O'Higgins 2012). Currently the only approved treatment for SCI is methylprednisolone, however, the efficacy of this treatment has been under scrutiny for the past decades (Hugenholtz 2003; Fehlings 2001; Bracken et al. 1997; Bracken et al. 1992; Bracken et al. 1990). As a result, there is a critical need to develop an effective treatment regimen for SCI victims.

In our current study we have used a clinically relevant clip-compression model for SCI which closely mimics the pathophysiology of human SCI (Cheriyan et al. 2014; Rivlin and Tator 1978a; Poon et al. 2007). Our model is designed to represent the most common form of human SCI; impact (contusion) plus persistent compression, which typically occurs through burst fractures with bone fragments compressing the cord or through fracture-dislocation injuries (Choo et al. 2007; Dumont et al. 2001; Fehlings, Smith, et al. 2012). Given the complex pathophysiology of SCI, it is becoming increasingly appreciated in the SCI field that therapies which target just one component of the secondary injury cascade ultimately fail to promote a meaningful recovery of function (Kwon et al. 2011; Karimi-Abdolrezaee et al. 2010; Alluin et al. 2014; Marsh et al. 2011). Therefore, any therapy which is moved towards clinical trials needs to show efficacy in modulating multiple components of the secondary injury cascade.

CSPGs are viable targets for repairing the injured spinal cord as they play a multifaceted role in secondary injury mechanisms of SCI. This study has evaluated a clinically feasible

approach in targeting CSPGs signaling in SCI. We show that pharmacological inhibition of LAR and PTPσ can exert multiple beneficial effects in SCI including the inhibition of RhoA activity. RhoA is a popular therapeutic target given that it is a convergent inhibitory pathway for both CSPGs and myelin associated inhibitors (MAIs) (Yiu and He 2006; Duffy et al. 2009; Dergham et al. 2002; Monnier et al. 2003; Niederöst et al. 2002). RhoA activation occurs immediately following SCI (Dubreuil, Winton, and McKerracher 2003; Conrad et al. 2005) and has been implicated to negatively modulate multiple aspects of the secondary injury cascade including: 1) axonal regeneration (Chan et al. 2005; Monnier et al. 2003; Dergham et al. 2002; Fournier, Takizawa, and Strittmatter 2003; Ramer, Borisoff, and Ramer 2004); 2) cell death (Dubreuil, Winton, and McKerracher 2003); and 3) inflammation (Impellizzeri et al. 2012). Here we also indirectly demonstrate that the Rho/ROCK pathway may also be involved in limiting cell replacement after SCI through CSPG-LAR and CSPG-PTPσ signaling. Pharmacological inhibition of LAR and PTPσ receptors was able to limit RhoA activation *in vivo* which was associated with improved functional recovery (Lang et al. 2015), axonal regeneration (Lang et al. 2015), oligodendrocyte proliferation, and modulation of the inflammatory response towards an M2 response.

Recently, an open-label phase I/IIa clinical trial using Ba-210, a Rho A inhibitor, came to completion (McKerracher and Anderson 2013; Lord-Fontaine et al. 2008; Fehlings et al. 2011a). The study demonstrated that Ba-210 was able to promote some motor recovery, however the patient number in this study was small (McKerracher and Anderson 2013). Future trials are planned to establish further efficacy for this treatment (McKerracher and Anderson 2013). Our findings provide further support for this treatment strategy given that our ILP/ISP treatment also modulated the Rho pathway after SCI.

Clinical trials using neural stem cells to treat SCI patients have recently begun in Canada (Badner, Siddiqui, and Fehlings 2017). In this trial, fetal derived NPCs were transplanted into areas rostral and caudal to the injury site, a technique adopted from a preclinical study first developed by Karimi-Abdolrezaee and colleagues in a rat SCI model in 2006 (Karimi-Abdolrezaee et al. 2006a; Badner, Siddiqui, and Fehlings 2017). This phase I clinical trial demonstrated the safety of NPCs for SCI, however, there was only some modest improvement in sensory function (Badner, Siddiqui, and Fehlings 2017). These findings were not surprising, given that work by our group and others had already demonstrated that transplantation of NPCs into the injured spinal cord on its own only provide modest benefits (Badner, Siddiqui, and Fehlings 2017; Karimi-Abdolrezaee et al. 2010; Karimi-Abdolrezaee et al. 2006a). When transplanting NPCs into the sub-acutely injured spinal cord, only limited number of cells survive (Karimi-Abdolrezaee et al. 2006a). Long-term stem cell survival can be increased by improving the spinal cord microenvironment with a cocktail of growth factors (EGF, bFGF, PDGF-AA) (Karimi-Abdolrezaee et al. 2006a). Unfortunately, this strategy fails when transplanting into the chronically injured spinal cord in rats (Karimi-Abdolrezaee et al. 2010). Only when the spinal cord was pre-treated with ChABC 1 week prior to stem cell transplantation in combination with the growth factor cocktail were NPCs able to survive and integrate into the tissue (Karimi-Abdolrezaee et al. 2010). Our findings here uncover the mechanisms behind which CSPGs are negatively modulating NPCs and identify novel therapeutic targets for combinatorial NPCs therapies. These findings indicate that CSPGs appears to promote a pro-inflammatory M1 phenotype in the injured spinal cord which may potentially be another mechanism behind the limited therapeutic benefits of NPCs. Although more preclinical studies are needed to confirm this hypothesis, our current findings indicate how crucial it is to pre-condition the lesion microenvironment prior to NPCs therapies. In conclusions, dysregulation of

CSPGs is a long-lasting pathology after SCI with a multifaceted inhibitory impact on the repair processes including cell replacement. There is an unmet need to develop clinically-relevant strategies to target CSPGs in SCI. While the bacterial enzyme ChABC has been employed successfully to study CSPGs in preclinical studies for decades, its translation as a therapy into clinical trials is challenged by its instability and its enzymatic nature with potential off target effects. Systemic ISP/ILP therapy renders a feasible, targeted and less invasive strategy to inhibit CSPG effects in SCI with great translational potential for clinical trials. Accordingly, findings presented in this thesis aid in developing effective strategies to target CSPGs in SCI in the future. Moreover, although this work has focused on SCI, our findings would have broader applicability and can be exploited in pharmacological and stem cell-based therapies for other CNS conditions that are characterized by the upregulation of CSPGs such as brain injuries and multiple sclerosis.

Chapter 6: References

6.1 References

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