Exploring the neurotrophic effect of pleiotrophin on glial cells

by

Somnath Jagdishprasad Gupta

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

Doctor of Philosophy

Neuroscience

University of Alberta

Abstract

Recovery after stroke depends on the extent of neuronal regeneration and myelination of existing and newly differentiated neurons. Regeneration process is governed by various factors released from glial cells. During an injury, glial cells activate and form a glial scar surrounding the lesion area. These activated glial cells produce and release various factors, including Chondroitin sulfate proteoglycans (CSPGs), a component of the extra cellular matrix. CSPGs accumulate in the glial scar and can modulate activity of adjacent cells including neurons, oligodendrocyte precursor cells (OPCs), and microglia. CSPGs inhibit the growth of neurons and OPCs and inhibit the differentiation of OPCs to oligodendrocytes. In this thesis, we examined how the neurotrophic factor pleiotrophin (PTN) could counteract these inhibitory effects in neurons and glia. We first tested the hypothesis that PTN can enhance the growth of neurons even in the presence of CSPGs. Primary cortical neurons cultured on an inhibitory CSPGs matrix exhibited reduced neurite growth relative to control conditions, but growth was restored with PTN treatment even in the presence of the inhibitory matrix containing CSPGs. PTN induced a dose-dependent increase in neurite outgrowth with optimum outgrowth at 15 ng mL^{-1} PTN (114.8% of neuronal outgrowth relative to laminin control). The growth-promoting effect of PTN was blocked by inhibiting the activity of anaplastic lymphoma kinase receptor (ALK) using 10 nM alectinib (ALK receptor antagonist) (53.59 % of neuronal outgrowth relative to laminin control). Additionally, in the presence of CSPGs, neurite outgrowth was also restored by activating signaling downstream of ALK via the protein kinase B pathway (Akt pathway) using SC79 (an Akt activator), with optimum outgrowth at 5 μM mL^{-1} PTN (78.43 % of neuronal outgrowth relative to laminin control). Thus, these data suggest that PTN can restore the neurite outgrowth of neurons on the CSPGs matrix by activating the protein kinase B pathway through ALK receptor.

Neurons with their axons myelinated by oligodendrocytes demonstrate improved signal conduction efficiency, a crucial factor in the optimal functioning of the nervous system. Moreover, microglia, as key players in the brain's immune defense, play a pivotal role in supporting neurons by monitoring for abnormalities, clearing debris, and contributing to the overall health and homeostasis of the nervous system. During an injury, CSPGs in the glial scar greatly inhibit differentiation of OPCs to oligodendrocytes and contribute to the activation of microglia. Here, we found that PTN can modify the release of pro-inflammatory cytokines from microglia in the presence of CSPGs. In the absence of inflammatory stimulation with IFN γ, PTN reduced the expression of pro-inflammatory cytokines. In contrast, PTN potentiated the release of pro-inflammatory cytokines in the presence of inflammatory stimulus with IFN γ. Thus, the data suggest that PTN can induce the differentiation of OPCs to oligodendrocytes, enhance the inflammatory activity of microglia in the presence of IFN γ, and reduce pro-inflammatory cytokines from microglia in the absence of an inflammatory stimulus.

Astrocytes exert a crucial influence on neurons by maintaining homeostasis, providing metabolic support, and participating in synaptic regulation, contributing significantly to the overall well-being and function of the nervous system. During an injury, activated astrocytes release CSPGs to form the glial scar, but no study to date has measured the effect of PTN on astrocytes in the presence of CSPGs *in vitro*. Our data suggest that PTN induces the release of pro-inflammatory cytokines from astrocytes. To create an environment more closely approximating the *in vivo* condition, also examined the effects of PTN on mixed glial cultures consisting of astrocytes, microglia, and oligodendrocytes in the presence of CSPGs. We found that in the presence of CSPGs, PTN reduced the expression of pro-inflammatory cytokines from mixed glia. In contrast, the PTN showed a trend towards increased expression of pro-inflammatory cytokine during co-

incubation with an inflammatory stimulus. Overall, our data indicates that PTN plays a pivotal role in modulating the activity of neurons and glial cells in the presence of CSPGs, fostering an environment that promotes the regeneration of neurons, the differentiation of oligodendrocytes, and the reduction of neuroinflammation in a manner that is modulated by the inflammatory activation of these cells. Thus, PTN holds the potential to be considered as a treatment therapy to enhance the recovery process after brain injury.

Preface

This thesis is an original work done by Somnath Jagdishprasad Gupta. All animal research was conducted in accordance with Canadian Council on Animal Care guidelines. All animals use protocols were approved by the University of Alberta Animal Care and Use Committee.

Chapter 2 of this thesis is published in *Neuroscience Insights* by Gupta SJ, Churchward MA, Todd KG, Winship IR. Pleiotrophin Signals Through ALK Receptor to Enhance the Growth of Neurons in the Presence of Inhibitory Chondroitin Sulfate Proteoglycans. Neuroscience Insights. 2023 Jul 15;18:26331055231186993. Doi: 10.1177/26331055231186993. PMID: 37465214; PMCID: PMC10350765. I collected and analysed the data and wrote the manuscript. Dr. Ian R. Winship, Dr. Kathryn G. Todd and Dr. Matthew. Dr. A. Churchward supervised experimental design, analyses and assisted with manuscript composition.

Research conducted for this thesis is a part of collaborative research between Dr. Ian. R. Winship and Dr. Kathryn. G. Todd at the University of Alberta. Dr. K. G. Todd, Dr. I. R. Winship, Dr. M.A. Churchward and I collaborated for the experimental design for chapters 2 – 4. All the data collection and analyses in these chapters are my original work. Dr. K. G. Todd, Dr. I. R. Winship, Dr. M. A. Churchward assisted in analyses of data and formatting thesis.

Acknowledgement

The completion of this PhD thesis has been a transformative journey, one that has been made possible through the unwavering support, guidance, and mentorship of three exceptional individuals: Dr. Ian Winship, Dr. Kathryn Todd, and Dr. Bradley Kerr. Their collective wisdom, dedication, expertise, and their student-friendly approach have left an indelible mark on this research endeavour.

Dr. Ian Winship has been the bedrock of this academic expedition. His profound understanding of Neuroscience and his exceptional ability to unravel complex problems have continually challenged me to aspire to new heights in my research pursuits. Dr. Winship's keen insights, meticulous attention to detail, and unwavering commitment to excellence have shaped the trajectory of this thesis and significantly influenced my intellectual growth. His encouragement to explore unconventional paths of inquiry has been instrumental in broadening the scope of this work.

In parallel, I extend my heartfelt gratitude to Dr. Kathryn Todd, whose multidisciplinary expertise and commitment to bridging theory and practice have enriched the depth and breadth of this thesis. Dr. Todd's dedication to fostering interdisciplinary approaches and her innate ability to connect disparate ideas have been invaluable. Her feedback, which has been consistently thoughtful and constructive, has played a pivotal role in refining the conceptual framework of this research.

I would also like to express my gratitude to Dr. Bradley Kerr for his invaluable contributions to this work. Dr. Kerr's expertise in Neuroscience and his willingness to engage in deep discussions and provide critical feedback has been instrumental in shaping this research's

methodology and analytical approach. His insights have brought a valuable perspective that has enriched the overall quality of this thesis.

All the Neurochemical Research Unit members at the University of Alberta have been a constant source of encouragement, support and friendship. Within the Neurochemical Research Unit, Dr. Glen Baker is an essential figure, leaving behind a legacy characterized by remarkable scientific achievements and exceptional kindness. Dr. Mathew Churchward, your invaluable mentorship and depth of knowledge have been a constant source of inspiration and I am grateful for your unwavering support in the realm of science. I am grateful to Patricia Kent, who has dedicated significant effort to support numerous graduate students, including myself. My fellow lab mates at the Neurochemical Research Unit including Dr. John W. Paylor, Dr. Mischa Bandet, Eszter Szepesvari, Dr. Junqiang Ma, Christopher Tsui, Hossein Dosti, Sima Abbasi and Juliana who have shared their idea, work and lives with me. This work was only possible because of funding support provided by Alberta Innovates Health Solutions, Heart and Stroke Foundation of Canada, the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada.

Finally, I would like to express my heartfelt gratitude and recognition to my family for their constant love and motivation. My parents and wife have stood by me with affection and comprehension in all my endeavors. Their unwavering belief in me and their unwavering commitment as parents are the primary factors behind any of my accomplishments. Thank you for being the most exceptional parents in the world.

Table of contents

Abstract ..... **ii**

Preface ..... **v**

Acknowledgement ..... **vi**

Table of contents ..... **viii**

List of tables ..... **xiii**

List of Figures ..... **xiv**

List of Abbreviations ..... **xvi**

Chapter 1: Introduction **1**

Stroke **2**

Mechanism of neuroplasticity after stroke **4**

Neuronal regeneration 4

Neurogenesis in the cortex ..... **Error! Bookmark not defined.**

Fate of glial cells in stroke **7**

Astrocytes 7

Microglia 10

Oligodendrocyte precursor cells (OPCs) and Oligodendrocytes 12

Impact of ECM on remyelination after stroke **14**

HSPGs 14

Tenascin - C (TN – C) 15

Fibronectin 15

Collagens 16

Hyaluronan 16

Laminin 16

CSPGs 17

Strategies to overcome inhibitory activity of CSPGs 20

Inhibitors of GAG chain synthesis 21

Inhibitors of CSPG signaling 21

Cleaving of CSPGs 22

CSPG Modulation: Beyond Digestion 25

PTN functions and receptors 26

Thesis outline and aims 32

Chapter 2: Pleiotrophin signals through ALK receptor to enhance growth of neurons in the presence of inhibitory CSPGs 32

Chapter 3: Pleiotrophin modulates the effect of CSPGs on activity of microglia and OPCs 33

Chapter 4: Pleiotrophin modulates the effect of CSPGs on the activity of astrocytes and mixed glia 34

Chapter 2: Pleiotrophin signals through ALK receptor to enhance growth of neurons in the presence of inhibitory CSPGs 36

Abstract 37

Introduction 38

Materials and Methods 40

Matrices preparation: 40

Primary cortical neuronal culture: 40

Microscopy, fluorescent immunostaining and image analysis 41

Statistics 41

Results 42

Pleiotrophin induces neurite outgrowth in the presence of CSPGs 42

Pleiotrophin signals via ALK receptor on neurons 42

Activating the AKT pathway drives neurite growth 43

Discussion 43

Figures 46

Chapter 3 : Pleiotrophin modulates the effect of CSPGs on activity of microglia and OPCs. ……52

Introduction 53

Effect of neuronal activity on OPCs and oligodendrocytes 53

OPCs in injury 53

Effect of CSPGs on OPCs and oligodendrocytes 54

Effect of MMPs on OPCs, oligodendrocytes and microglia 55

Effect of PTN on OPCs 56

Effect of CSPGs on microglia 56

Effect of PTN on microglia 57

Materials and Methods 58

Matrices preparation: 58

Primary OPC cell culture: 58

Primary microglia cell culture 60

EdU proliferation assay 60

Gel zymography 61

Immunocytochemistry 62

Statistics 62

Results 63

PTN drives the differentiation of OPCs to oligodendrocytes, overcoming the inhibitory effect of CSPGs 63

Effect of PTN on process outgrowth from OPCs and oligodendrocytes. 65

Effect of PTN on proliferation and release of pro-inflammatory cytokines from microglia in the presence of CSPGs 67

Effect of PTN on proliferation and release of pro-inflammatory cytokines from microglia in the presence of CSPGs under inflammatory stimulus 68

Expression of MMPs from PTN-treated microglia in the presence of CSPGs 69

Discussion 71

PTN overcomes CSPGs' inhibitory effects on differentiation of OPC 71

PTN induces complex cellular outgrowth process of OPCs and oligodendrocytes. 72

PTN reduces the release of pro-inflammatory cytokines and MMP in the presence of CSPGs 73

PTN restores inflammatory conditions from microglia in the presence of CSPGs under inflammatory stimulus..... 74

Conclusion:..... 75

Figures 77

Chapter 4: Pleiotrophin modulates the effect of CSPGs on the activity of astrocytes and mixed glia..... 97

Introduction 98

Materials and Methods 100

Matrices preparation:..... 100

Primary astrocytes cell culture: 100

Primary mixed glia cell culture 102

Cytokines analysis..... 103

Gel zymography 103

Immunocytochemistry..... 103

Statistics 104

Results..... 104

Effect of CSPGs on morphology of astrocytes 104

Effect of PTN on the release of pro-inflammatory cytokines from astrocytes in the presence of CSPGs 105

Effect of PTN on the release of MMP from astrocytes in the presence of CSPGs..... 106

Effect of PTN on the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs..... 107

Effect of PTN on the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs during inflammatory stimulus 108

Effect of PTN on the expression of MMPs from mixed glia in the presence of CSPGs 109

Discussion 109

PTN activates astrocytes and induces the release of pro-inflammatory cytokines 109

PTN modulates the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs. 110

PTN reduces the release of MMPs in the presence of CSPGs..... 111

Conclusion **112**

Figures **114**

Chapter 5 : Summary, conclusion and future work **132**

Neurogenesis and neuritogenesis 133

Axonal growth and myelination 134

Neuroinflammation 135

Angiogenesis 136

PTN restores the growth of neurons in the presence of CSPGs 136

PTN induces differentiation of OPCs to oligodendrocytes in the presence of CSPGs 138

PTN modulates the expression of cytokines and MMPs from microglia in the presence of CSPGs 138

PTN modulates the expression of pro inflammatory cytokines from astrocytes and mixed glia 140

References **146**

List of tables

Table 1: Different sulfation patter on GAG side chains in CSPGs..... 18

Table 2: Radius process outgrowth from PDGFR α, O4 and MBP positive cells showing significant difference in number of intersections with PTN treatment..... 66

List of Figures

Figure 1.1: Summarized effects of PTN and CSPGs on neurons and glial cells 31

Figure 2.1: Effect of PTN on the neurite outgrowth in the presence of CSPGs 46

Figure 2.2: Effect of alectinib on the neurite outgrowth in the presence of CSPGs 48

Figure 2.3: Effect of SC79 on the neurite outgrowth in the presence of CSPGs 50

Figure 3.1: PTN induces expression of various oligodendrocyte lineage differentiation marker. 79

Figure 3.2: Effect of PTN on differentiation of OPCs to oligodendrocytes 81

Figure 3.3: PTN promotes cellular process outgrowth from oligodendrocytes and OPCs. 83

Figure 3.4: PTN treatment reduced the release of pro-inflammatory cytokines and proliferation of microglia. 85

Figure 3.5: PTN treatment promoted the release of pro-inflammatory cytokines and proliferation of IFN γ stimulated microglia. 87

Figure 3.6: PTN treatment promoted the cleavage of CSPGs by microglia. 90

Figure 3.7: PTN treatment induced the abundance of MMP 9 and MMP 2 from microglia 92

Figure 3.8: Effect of PTN treatment on abundance of MMP 9 and MMP 2 from IFN γ stimulated microglia 94

Figure 3.9: Summarized effect of PTN on OPCs and microglia in the presence of CSPGs 95

Figure 4.1: Purity of astrocytes by immunofluorescence 114

Figure 4.2: Astrocytes cultured on CSPGs matrix. 115

Figure 4.3: PTN treatment induced the release of pro-inflammatory cytokines from astrocytes. 117

Figure 4.4 PTN treatment reduced the release of MMPs from astrocytes in the presence of CSPGs. 120

Figure 4.5: PTN treatment reduced the release of pro-inflammatory cytokines from mixed glia. 122
Figure 4.6: Effect of PTN treatment on the release of pro-inflammatory cytokines from mixed glia under inflammatory stimulus 124
Figure 4.7: PTN treatment reduced the release of MMP 9 from mixed glia. 127
Figure 5.1: Summary of effect of PTN on neurons, OPC, astrocytes and microglia..... 143
Figure 5.2: Potential role of PTN during recovery after stroke 144

List of Abbreviations

TBI, Traumatic Brain Injury,

ATP, Adenosine Triphosphate;

tPA, tissue plasminogen activator;

CNS, central nervous system;

AQP4, aquaporin 4;

DAMPs, damage-associated molecular patterns;

BBB, blood-brain barrier;

IFN γ, interferon γ;

VCAM-1, Vascular Cell Adhesion Molecule-1;

ICAM -1, Intercellular Adhesion Molecule-1;

NGF, nerve growth factor;

VEGF, vascular endothelin growth factor;

GDNF, glial-derived growth factor;

EPO, erythropoietin;

CSPGs, chondroitin sulfate proteoglycan;

TLR, Toll-like receptors;

IGF, Insulin-like growth factor;

BDNF, brain-derived neurotrophic factors;

TGFβ1, transforming growth factor-β1;

OPCs, Oligodendrocyte precursor cells;

PDGF AA, platelet-derived growth factor AA;

FGF, fibroblast growth factor;

ET -1, Endothelin 1;

EGF, epidermal growth factor;

ErK1/2, Extracellular signal-regulated kinase ½;

PTN, Pleiotrophin;

NMDA, N-methyl-D-aspartate;

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;

MHC I, Major Histocompatibility Complex;

ECM, Extracellular Matrix;

HSPGs, Heparan sulfate proteoglycans;

TN C – C, Tenascin-C;

GAG, Glycosaminoglycan;

GlcA, glucuronic acid;

GlcNAc, N-acetyl glucosamine;

PNN, perineuronal nets;

NG2, neuroglial antigen 2;

PTP σ, Protein Tyrosine Phosphatase Sigma;

LAR, Leukocyte common antigen-related;

Ng R, Nogo R;

TLR, Toll-Like Receptor;

TNF, Tumour Necrosis Factor;

MS, Multiple sclerosis;

drNPC, directly reprogrammed human neural precursor cells;

ChABC, Chondroitinase ABC;

SCI, Spinal cord injury;

MMPs, Matrix Metalloproteinases;

NSC, Neural Stem Cells;

SVZ, Subventricular Zone;

SGZ, Subgranular Zone;

PTP, Protein tyrosine phosphatase;

rmPTN, recombinant human PTN;

rhPTN, recombinant mouse PTN;

MAP 2, microtubule-associated protein 2;

JAK, Janus kinase;

PTPRZ, protein tyrosine phosphatase receptor type Z;

Dnase, Deoxyribonuclease;

MBP, myelin Basic protein;

MCP 1, Monocyte chemoattractant protein 1;

LPS, Lipopolysaccharide;

DMEM-F12, Dulbecco modified eagle medium/ham's F12;

FBS, Fetal Bovine Serum;

TNF, tumor necrosis factor;

IL-1β, interleukin-1 beta;

IL-6, interleukin-6;

IL-10, interleukin-10;

IL – 12p70, Interleukin-12p70;

PLL, Poly L Lysine;

PDL, Poly D Lysine

Chapter 1: Introduction

Modulators of neuroplasticity after stroke

The brain is the command center of human body. It is a complex organ consisting of billions of electrically conductive cells known as neurons and other cells known as glial cells that provide support and nutrients for the normal functions of the brain. This organ is responsible for governing our thoughts, memories, emotions, decisions, and actions. Understanding brain structure and function is fundamental to find treatments for brain injury or disease. Brain damage results from the death of injured cells and inflammation [1]. Common causes of brain injury include mechanical damage that results in traumatic brain injury (TBI) and insufficient supply of blood to the brain that results in stroke. Our primary interest is in the modulation of neuronal structure and function to counteract death and disability due to stroke. To that end, in this chapter, I synthesize information about occurrence of stroke and available treatment strategies and their shortcomings, with a goal of identifying possible alternative treatment strategies.

Stroke

Stroke refers the damage and dysfunction in the brain caused by insufficient blood supply. Ischemic stroke accounts for approximately 85% of stroke cases and occurs due to an obstruction in a blood vessel supplying blood to the brain. 15% of stroke cases are classified as hemorrhagic strokes, which occur due to rupture of blood vessels in the brain [2,3]. Most individuals who have experienced a stroke are left with enduring brain damage, rendering it the primary contributor to acquired sensory-motor disability among adults [4]. About 94% of stroke survivors may encounter sensory-motor impairments following a stroke, resulting in impaired motor activities even following rehabilitation efforts [5].

In ischemic stroke, the viability of neurons depends on their location relative to the degree of ischemia. The area where blood supply is reduced most severely is known as the ischemic core.

The tissue surrounding the ischemic core is termed as penumbra [1]. The ischemic core develops when the blood supply is severely impaired, which leads to an insufficient supply of oxygen and glucose to the cells in that area. The inability to perform oxidative phosphorylation sufficient for ATP generation leads to necrotic cell death within minutes of ischemic onset [6] . The penumbra refers to a surrounding region of brain that is functionally silent but viable, as it has reduced but not a complete cessation of blood flow. This generates hypoxic environment that limits the metabolic activity of neurons, causing injury but still maintaining cellular viability [7, 6]. However, the survival of the neurons in penumbra is time-dependent, and if the blood supply is not restored the neurons will die within a few hours to days [1].

The existing strategies for treating stroke include the use of thrombolytic drugs such as tissue plasminogen activator (tPA), which when administered intravenously within 4.5 hours of stroke can restore perfusion by lysis of the clot that is causing vessel occlusion [8]. Limitations associated with the use of tPA for stroke treatment include use only for ischemic stroke, and the 4.5-hour window for treatment, [9] which means many patients are not eligible for treatment. After 4.5 h, the risk of hemorrhagic transformation outweighs potential benefit [10]. EVT (mechanical thrombectomy) is a newer treatment for severe strokes due to large vessel occlusion. While major improvement in stroke outcomes can be achieved, the majority of EVT treated patients still have major persistent disability at 90 days [11–13]. Without additional effective treatments, most survivors of stroke will continue to have permanent disability after ischemic stroke [10].

Brain damage due to ischemia can be reduced by increasing the blood flow to the penumbra to restore the supply of nutrients to damaged cells. However, even with successful restoration of flow some brain damage still occurs. The brain has endogenous mechanisms of neuroplasticity to aid in recovery from such damage. Neuroplasticity is a physiological process

in which neural networks reorganize by either forming new synaptic connections or modifying the strength of existing connections [14 -15]. There are several significant neuroplasticity related events associated with functional recovery, including (a) the activation of parallel pathways, which helps restore impaired function; (b) the generation of new neurons; and (c) the initiation of the growth in existing neurons in penumbra [16].

Mechanism of neuroplasticity after stroke

Neuroplasticity results in alterations in the organization of neural components that occur within the central nervous system over the course of an individual's entire life. These modifications are greatly involved in the process of learning and adapting to a new environment. Moreover, this dynamic nature of neurons, to change their activity in response to any stimuli (internal or external) encourages to reorganize the neural network structure after any brain injury. Neuroplasticity in the brain following a stroke allows for the adaptation of neural networks, leading to both physiological and anatomical changes [17]. These adaptations facilitate functional recovery by repairing or restoring damaged circuitry [17]. The surviving neurons establish connections with other neurons, enabling them to strengthen the existing network or substitute for impaired networks and thus restore functional recovery. This process permits the unaffected neurons in the nervous system to acquire functions from damaged areas.

Neuronal regeneration

Neurogenesis in the cortex

In the adult brain, new neurons are generated by differentiation of neuronal progenitor cells (NPCs) from two specialized regions in the brain: (1) the subventricular zone (SVZ); and (2) the subgranular zone (SGZ) [18]. During the initial 2 weeks after stroke, there is an increased

proliferation of NSCs in SVZ and SGZ [19]. After proliferation, these immature neurons migrate to the damaged area of the brain and can differentiate to mature neurons [20]. Several factors govern the differentiation and migration process of NPC which includes Transforming growth factor alpha (TGF α), induced the expression of nestin in NPCs, induced proliferation, migration and differentiation of NPCs in ischemic region [20]. Neurotrophic factors like brain derived neurotrophic factor (BDNF), ciliary neurotrophic factors (CNTF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) induce proliferation of NPCs and promotes neuronal survival after stroke [21,22].

Synaptic plasticity

Formation of new synaptic connections during development are facilitated by guidance of growing axons by various extracellular matrix components (ECM) [23]. During the recovery process after stroke or other neurotrauma, there are factors released that potentiate and inhibit the regeneration of neurons, including processes related to axonal growth. Regeneration of axons is greatly inhibited by 2 major components: (1) myelin associated inhibitors (MAI) and (2) Chondroitin sulfate proteoglycan (CSPGs) [23,24].

Myelin Associated Inhibitors (MAIs)

MAIs are proteins synthesized by oligodendrocytes, serving as integral components of myelin in the central nervous system (CNS). These MAIs exert inhibitory effects on neurite outgrowth in vitro and are presumed to constrain axon growth *in vivo* following CNS injuries. Several studies have shown that MAIs include Nogo A, semaphorin 4D, oligodendrocyte myelin glycoprotrein (OMgp) and myelin associates glycoprotein (MAG) [23]. Out of these MAI Nogo A, MAG and OMgp interact with neuronal Ng R1 receptor and inhibit the regeneration of axons by inducing growth cone collapse [25]. In mouse spinal cord injury (SCI) models, genetic ablation of

Nogo A not only restored the corticospinal and raphe-spinal tract but also improved functional recovery compared to sham group [23]. Antibodies against Nogo-A have been shown to promote neurite outgrowth *in vitro* and improved functional recovery after CNS injury [26,27]. The timing of administration of these antibodies also has an important role in their effects. When delivered prior to motor training, blocking Nogo-A improves motor deficits, but when given during motor training it is not effective at improving motor deficits [26,27]. MAG is an inhibitory protein present in the CNS that can interact with various receptors including Nogo receptor R1 (Ng R1), Nogo receptor 2 (NgR2) and Pir B [25]. *In vitro,* studies have shown reduced axonal outgrowth from dorsal root ganglion cells (DRG) when cultured on MAG expressing cells, with growth restored upon treatment with anti-MAG antibodies [28].

Chondroitin sulfate proteoglycans (CSPGs)

The glial scar that develops surrounding the injury site acts as a physical barrier to regeneration. The main components of glial scar are CSPGs, which are released by reactive astrocytes and microglial cells after CNS injury [29]. CSPGs bind to receptor protein tyrosine phosphatase sigma (PTP σ) and leukocyte antigen receptor (LAR) and activate Rho A and ROCK pathway which negatively affects actin polymerisation and causes growth cone collapse [29]. Several approaches have been implemented to overcome the inhibitory activity of CSPGs by (1) inhibiting the downstream signalling using RhOA and ROCK inhibitors; (2) Applying chondroitinase ABC (ChABC) to cleave CSPGs; (3) Blocking intracellular signals using intracellular peptides. All these approaches have shown positive effect on the growth of axons by overcoming the inhibitory effect of CSPGs [30–33].

Although there is an upregulation of factors inhibiting plasticity after stroke or an injury, there is also an upregulation of factors that initiate the recovery process and induce plasticity.

Expression of growth-promoting genes varies from early stages to later stages of injury [34]. During the early stage, small rich proline rich protein (SPRR1) gets upregulated and is known to promote axonal growth [35], followed by p21, tubulin and Myristoylated alanine-rich C-kinase substrate (MARCKS) [22]. MARCKS potentiates axon development by engrafting plasmalemmal precursor vesicle carrying component necessary for extension of axon plasma membrane [36]. The growth promoting genes upregulated during late stage include SCG10 Like protein (SCLIP), which plays an important role in regulating microtubule cytoskeleton within axons [37]. Several proteins are consistently expressed during active recovery including GAP43, CAP 23 and c-Jun [34], all of which are associated with axonal growth and development [34–37]. Thus, to achieve a better recovery after stroke, upregulating the expression of growth inducing proteins and overcoming the effect of inhibitory factors is essential.

Fate of glial cells in stroke

While most studies have focused on the effects of ischemia on neurons, other cells that play a significant role in promoting the healthy state of neurons are glial cells. There are 3 major types of glial cells: (a) astrocytes, which provide metabolic and mechanical support to neurons; (b) Oligodendrocytes, which provide support and insulation to axons in the CNS and (c) microglia, the immune cells in the brain which provides protection against infection and helps clearing the debris and dead cells. Brain injury including stroke and neurodegenerative disease exert a profound influence on the function of glial cells within the CNS, altering their morphology, proliferation rates, and gene expression patterns.

Astrocytes phenotypes in stroke

Astrocytes in CNS play a vital role in maintaining the healthy state of neurons by providing metabolic and functional support to neurons. Astrocytes help to maintain extracellular K^+

homeostasis, provide metabolic support and uptake excess glutamate and neurotransmitter to keep normal functioning of neurons [38]. During stroke, neuronal and astrocytic activity becomes impaired within 30 min of ischemic onset, affecting functions including buffering of K^+ and glutamate uptake, which leads to increased neurotransmitter release and excitotoxicity to neurons [5]. Following ischemia, the astrocytes become reactive (RAs). *In vitro* activation studies have characterized RAs to be of A1 phenotype or A2 phenotype. A1 phenotype is associated with reactive or pro-inflammatory state while A2 phenotype is associated with anti-inflammatory and neuroprotective [39]. However, *in vivo* RAs are localized in penumbra and can be divided into 7 different subgroups [40]. A major signalling pathway activated in pro inflammatory astrocytes is NF-κB, upregulating the expression of inflammatory cytokines and complement system proteins [40]

Astrocytes and inflammation during stroke

Astrocytes play a major role in reducing oxidative stress by providing glutathione to neurons [41]. During ischemic conditions, RAs showed an increased release of reactive oxygen species (ROS), nitric oxide (NO), peroxynitrite and superoxides inducing oxidative stress [42] and decrease in glutathione synthesis, this decrease in glutathione leads to neurotoxicity [39,43]. RAs modulate inflammation by releasing various inflammatory factors. RAs releases cytokines including interleukins (IL 6 and IL 1 β), transforming growth factor β (TGF β), Tumor necrosis factor α (TNF α) and interferon γ (IFN γ) [44]. Additionally, RAs show an increased expression of IL 17 receptor and binding with IL 17 released from infiltrating T cells, which induces the release of pro inflammatory cytokines [40]. RAs also upregulate the expression of various cell adhesion molecules including Vascular Cell Adhesion Molecule-1 (VCAM-1), Platelet-selectin (P-selectin) and Intercellular Adhesion Molecule-1 (ICAM -1) which increases the infiltration of leukocytes and platelets contributing to increased inflammation [45]. Thus, the inflammatory cytokines and ROS

generated during ischemic events can directly or indirectly induce neuroinflammation leading to the death of neurons.

RAs also promote the release of various anti-inflammatory factors that can inhibit the inflammatory response. RAs in a mouse stroke model induce the release of brain dopamine neurotrophic factor (BDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) reducing the release of inflammatory cytokines by alleviating endoplasmic reticulum (ER) stress [40]. TGF β signalling in astrocytes reverts the inflammatory effect induced by IL 1 α, TNF α, and c1 q [46]. Additionally, RAs upregulate the release of various neurotrophic factors including NGF, vascular endothelin growth factor (VEGF), GDNF, and erythropoietin (EPO) [4547]. Growing evidence also suggests that activated astrocytes show an increase in the expression of PTN when activated [48 49]. Thus, RAs can induce both neuroprotective and neurotoxic characteristics.

Astrocytes and ECM

Astrocytes influence the extracellular environment by releasing soluble factors including cytokines and growth factors but can also modulate the response of different cells in CNS by expressing components of the ECM. The ECM is a network of proteins and glycoproteins into that surrounds cells of the CNS. This interwoven network consists of HSPGs, CSPGs, keratan sulfate proteoglycans, laminin, fibronectin, tenascin C (tn C) and collagens [47]. Astrocytes in ischemic conditions undergo reactive gliosis. These RAs are characterized by increased glial fibrillary acid protein (GFAP) [50 39] and are involved in the formation of a glial scar around a site of injury [477]. A glial scar consists of RAs, activated microglia and ECM released from activated glial cells that surround the ischemic core [15 50]. The glial scar serves as a physical barrier to restrict the spread of damage to other parts of the brain, thus serving a beneficial role [7]. Mice that do not express GFAP and vimentin have impaired formation of the glial scar [39] with increased bleeding and more debris

at the site of injury [51]. One of the major components of glial scar is CSPGs. RAs upregulate the release of CSPGs during ischemia [50, 52 53]. CSPGs are known inhibitors of neuronal growth and OPC differentiation [29,54], inhibiting the regeneration of neurons and myelination of regenerated neurons. Thus, while the glial scar is protective early after injury, identifying ways to block CSPG signalling or synthesis can help overcome the inhibitory activity of CSPGs in regeneration process.

Microglia

Microglia are the resident CNS-specific immune cells and play a major role in neuroinflammation. After ischemia, microglial interactions with synapses are increased, and this is subsequently accompanied by the disappearance of the presynaptic bouton [55]. Damaged cells in the infarct area release DAMPs which can interact with microglia and initiate an immune response [56]. These DAMPs include various proteins, DNA, ATP, and other metabolites which can interact with receptors including Toll-like receptors (TLR), glutamate receptors and purinergic receptors on microglia [57, 58 59]. Once activated, these receptors trigger an upregulation in the expression of pro inflammatory cytokines IL 6, IL 1 β, TNF, and NO [60 61].

Microglia and neuroinflammation

Activation of microglia can be approximated by characterization into pro inflammatory and anti inflammatory phenotypes. Based on *in vitro* studies, stimulation with IFN γ induces pro inflammatory phenotype releasing of pro inflammatory cytokines, including IL 1 β, IL 6, TNF and ROS [62]. The hallmark protein markers associated with pro inflammatory phenotype include CD 11b, CD16, CD 32, and CD86 [63]. Stimulation with IL 4/IL 13 induces anti inflammatory phenotype [64] with upregulation in the release of IL 4, IL 10 and major histocompatibility complex II (MHC II) [65,66]. Microglia with anti inflammatory phenotype are associated with tissue regeneration, clearing the cellular debris and production of extracellular components [66]. Microglia with anti

inflammatory phenotype are also associated with the release of various neurotrophic factors: IGF, BDNF, and NGF [65]. Notably, characterization of microglia based on *in vitro* studies might not mimic the activation *in vivo* during stroke, where a wide range of factors released from a heterogenous cell population [63] can modulate microglia, and therefore this classification is an oversimplification.

Microglia in stroke

In mice administered the photothrombotic (PT) stroke model, the expression levels of microglial markers CD16 and CD32 were observed to increase on day 1 and remained elevated through day 14 [66]. Similarly, several inflammatory cytokines, including TNF-α, IL 1β, and IL 6, continued to be highly expressed in the ischemic area at days 14 and 21 post PT stroke [66]. In transient middle cerebral artery occlusion (tMCAo) mouse models, markers for both anti inflammatory and pro inflammatory microglia are observed from day 1 to day 14 [67]. Microglia with anti inflammatory marker expression initiates from 24 h, peaks at day 5, and declines by day 7 [68,69]. The expression of markers shifts from anti inflammatory to pro inflammatory from day 2 – day 7 near the infarct core, and the expression of pro inflammatory markers continues till day 14 [68]. During the early stage of stroke, when anti inflammatory phenotype dominates the core and penumbra, the release of TGFβ1 activates astrocytes releasing excess CSPGs in the extracellular matrix to form the glial scar, which is essential to restrict the spread of damage to other parts of brain [70]. Although the above-mentioned data classifies microglia based on activated gene profile, single cell RNA sequencing from reactive microglial cell populations has shown that microglia co-express genes from both polarized states [71]. Thus, classifying microglia in different phenotypes can mislead the interpretation of findings especially for studies like brain injury where it has been shown that the microglia are present as mixed phenotypes in a complex environment. Overall, in

the penumbra region following an ischemic event, microglia undergo heightened activation, proliferation, and migration, primarily to repopulate the core region. Over the course of months after post-ischemia, there is prolonged activation of microglia in both the penumbra and the unaffected tissue, which can ultimately result in secondary neuronal damage [72].

Oligodendrocyte precursor cells (OPCs) and Oligodendrocytes

OPCs are glial cells that play a major role in myelination in CNS. OPCs proliferate and differentiate into mature oligodendrocytes, extending their processes to wrap around and myelinate axons [73]. OPCs maintain homeostasis between proliferation and differentiation to mature oligodendrocytes to ensure the maintenance of myelination [74].

Several factors govern the proliferation of OPCs, including platelet-derived growth factor AA (PDGF AA), which is released from neurons and astrocytes and promotes the proliferation of OPCs [75, 76]. Further, *in vitro* studies have shown that fibroblast growth factor (FGF) and epidermal growth factor (EGF) are also essential for the proliferation of OPCs [77]. Moreover, endothelin 1 (ET -1) maintains the proliferative state of OPCs, by activating Ras and p38 pathway and inhibiting signalling induces the differentiation of OPCs to oligodendrocytes [76]. *In vitro,* studies have shown that blocking extracellular signal-regulated kinase 1/2 (ErK1/2) signalling pathway blocks the proliferation of OPCs [78].

The differentiation process for oligodendrocytes includes changes in size of OPCs and upregulation of membrane lipid synthesis [79]. Internal and external factors modulate the differentiation of OPC to oligodendrocytes [80]. External factors include proteins released from reactive microglia and astrocytes in CNS, which include neuregulin, CNTF, BDNF, PTN, and thyroid hormone (TH) [76]. Notably, glutamate signalling and neuronal activity can dictate the migration, proliferation and differentiation of OPCs. Neurons with higher neuronal activity

increase differentiation of local OPCs [76,81]. However, in some neurons activation of glutamate receptors inhibits the proliferation of OPCs and favours MBP translocation. In GABAergic neurons, signalling through $GABA_A$ inhibits the proliferation and differentiation while signalling through $GABA_B$ receptor stimulates differentiation and migration [82].

Effect of ischemia on OPCs proliferation and differentiation

New oligodendrocytes are required to remyelinate the existing neurons after an injury to restore the efficient conduction of nerve impulses. During ischemic stroke, oligodendrocytes are susceptible to damage due to ischemia, thus leading to loss of myelination around neurons and impairing neuronal function [83]. After a stroke, TGF β released from activated glial cells induces migration of OPCs to injured site [84,85]. In the lysolecithin model of demyelination, OPCs are generated from neural progenitor cells of SVZ [86–88]. ROS generated due to an injury induces oxidation of lipids and DNA damage in oligodendrocytes [89]. OPCs express both NMDA and AMPA receptors, and damaged neurons and activated astrocytes release glutamate in the extracellular environment, leading to glutamate toxicity [90]. *In vitro* studies have shown that AMPA receptor antagonists can reduce the hypoxia induced injury to oligodendrocytes [88,90].

Effect of cytokines on OPCs proliferation and differentiation

Cytokines also affect the differentiation and proliferation of OPCs. IFN γ released from activated glial and infiltrating T cells reduces OPC differentiation and induces ER stress, eventually leading to apoptosis [91]. Oligodendrocytes also function as antigen-presenting cells, with upregulation of genes associated with the expression of MHC I [85,88]. Cytokines including IL 6 and IL 17 promote differentiation, while IL 11 and IL 1β promote the survival of oligodendrocytes [84,88]. Insulin like growth factor (IGF 1) released from astrocytes promotes differentiation, while bone morphogenic proteins inhibits the differentiation process. Growth factors including PDGF,

FGF and VEGF promote proliferation of OPCs and BDNF promotes both proliferation and differentiation [85].

Impact of ECM on remyelination after stroke

Plasticity after stroke depends on the extent of regeneration of neuronal connections and their myelination. The ECM consists of various molecules released from cells that shape the growth of neurons, guides synapse formation and myelination of neurons. The main components of ECM all have essential roles in regulating remyelination process, as highlighted below.

HSPGs

HSPGs consist of a core protein with repeating disaccharide units of N-sulfated glucosamine (GlcNs) and Iduronic acid (IdoA) [92,93]. Membrane-bound HSPGs include syndecans, glypicans, neuropilin-1 and betaglycan, and extracellular basement membrane includes argin, perlecan, and argin [92]. HSPGs have diverse effects on neurons. Membrane-bound HSPGs interact with various signaling molecules including fibroblast growth factor (FGF), WNT, hedgehog, and transforming growth factor β (TGF β) [94]. HSPGs bind to receptor protein tyrosine phosphatase sigma (RPTPσ), inhibiting phosphatase activity and potentiating axonal growth [95]. SDC2 contributes in the maturation of synapse by interacting with FGF22 and increasing the accessibility to postsynaptic FGF22 receptor [94,96]. Similarly, interaction of released astrocytic glypican 4 (GPC4) with RPTP σ induces release of neuropllin1 which activates AMPAR and forms active synapse [94.]

HSPGs may modulate oligodendrogenesis by regulating the extracellular environment. Oligodendrocytes exhibit varying levels of HSPG expression as they progress through different stages of differentiation [97]. Expression of HSPGs is upregulated in astrocyte and neurons during an injury [98], increased syndecan 2 and 4 expression favour differentiation while syndecans 1 and

3 favours FGF-mediated proliferation [97] by accumulating more FGF 2 and PDGF thus inhibiting the differentiation of OPCs.

Tenascin - C (TN – C)

TN-C is a glycoprotein expressed by radial glial cells during early CNS development, and in adult, its expression is limited to astrocytes in astrocytes of SVZ . The limited literature indicates that TN – C regulates the migration of neural stem cells during the process of differentiation. TN - C inhibits the retinal neurite outgrowth [99]. Interestingly, although TN - C is associated with the above mentioned effects on neurons, knock out mice do not show any abnormal neural development [100].

TN-C interacts with various cell membrane receptors, including integrins, TLR4, and epidermal growth factor receptor (EGFR) activating MAPK pathway [101]. TN-C has binding affinity towards other ECM molecules like lectican CSPGs (brevican, neurocan, aggrecan and versican) and cytokines like PDGF, transforming growth factor β (TGF-β), insulin-like growth factor binding proteins, and heparin [101,102]. The expression of TN - C is upregulated during an injury and is known to inhibit the migration of OPCs to TN-C rich areas [92]. Primary OPCs cultured in the presence of TN-C showed reduced expression of MBP, thus suggesting that TN-C inhibits the migration and differentiation of OPCs to oligodendrocytes [92] .

Fibronectin

Fibronectin is a glycoprotein produced from astrocytes, epithelial cells fibroblast and mesenchymal cells [97]. Fibronectin promotes neurite outgrowth in developing CNS and peripheral nervous system (PNS) [103]. Expression of fibronectin is upregulated by reactive astrocytes and activated microglia in the lesion area [104]. Fibronectin inhibits the differentiation of OPCs to oligodendrocytes, and reduces outgrowth of OPCs, but does not inhibit the migration of OPCs [97].

Collagens

Collagens are polypeptides and can be divided into 28 types (I – XXVIII) based on their molecular structure[97]. In the CNS, under normal conditions of homeostasis, collagens I, III, and IV can be found within the glia limitans externa and the basement membrane of blood vessels [105]. Collagen type I promotes neurite outgrowth in vitro, while collagen IV present in the extracellular matrix inhibits the neurite outgrowth [106]. Following injury, collagens I and IV are deposited into the damaged area and become significant constituents of the glial scar tissue [105,107]. Effects of collagen on OPC differentiation and proliferation is not well studied. *In vitro* studies indicate that collagen reduces the migration of OPCs and collagen IV inhibits the growth of premyelinating oligodendrocytes [97].

Hyaluronan

Hyaluronan, a linear unsulfated glycosaminoglycan (GAG), consists of alternating units of glucuronic acid (GlcA) and N-acetyl glucosamine (GlcNAc) [108] that is secreted from astrocytes. Hyaluronan is the basic component of perineuronal nets (PNN) around neurons and can interact with the N-terminus of core proteins of lectican family CSPGs [108]. Hyalauronan can promote neurite outgrowth, protect neurons from oxidative stress, inflammation and excitotoxicity [109]. Hyaluronan modulates the synaptic plasticity in neurons thus affecting memory and learning [110]. During stroke or brain injury the reactive astrocytes release high molecular weight hyaluronan and is deposited in the glial scar lesion area and is known to inhibit the maturation of OPCs thus affecting remyelination [97].

Laminin

Laminins are high molecular weight glycoproteins present in the extracellular matrix. Laminin promotes the neurite outgrowth, guides neurons to form stable connections and modulates

synapse formation [111–113]. During an injury, the reactive astrocytes release laminin and gets deposited at lesion site [114,115]. Laminin increased the survival of OPCs and promotes the differentiation to mature oligodendrocytes [115].

CSPGs

CSPGs are released from astrocytes, oligodendrocytes and neurons [116,117]. CSPGs can be classified into

- lecticans family - aggrecan, versican, neurocan and brevican.
- phosphocan and neuroglial antigen 2 (NG2) [118].

CSPGs consist of a central core protein with GAG side chains containing N-acetylgalactoseamine (GalNAc) and glucuronic acid (GlcA) with varying sulfation patterns. This configuration enables CSPGs to engage with neuronal receptors [119]. All CSPGs except NG2 have immunoglobulin-like domain in the protein structure except NG2 which has 2 extracellular domain separated by GAG chains [119]. Three factors dictate the biological activity of individual proteoglycans:

1) the length of the core protein
2) the number of GAG side chains attached to the central domain.
3) the sulfation patterns of N-acetylgalactose amine of GAG side chains. GAG exist is in different sulfation pattern as described in Table 1:

Table 1: Different sulfation patterns on GAG side chains in CSPGs

Monosulfation	CS A	N-acetylgalactosamine on the axial 4 carbon
	CS C	N-acetylgalactosamine on the equatorial 6 carbon
Disulfation	CS D	carbon 2 N-acetylgalactosamine and carbon 6 of glucuronic acid
	CS E	4 and 6 of N-acetylgalactosamine

CS B consists of hybrid dermatan sulfate/chondroitin chains with sulfation on carbon 4 of N – acetylgalactosamine and carbon 4 of iduronic acid [120,121].

CSPGs and neurons

CSPGs can exhibit either inhibitory or permissive effects on axonal growth depending on the specific molecular position of sulfation within the disaccharide unit [118,122]. Proteoglycan with CS – A inhibit axonal outgrowth, while the effect of CS – C can induce both growth permissive and inhibitory effects on axonal growth [121]. Moreover, *in vitro* studies have shown that CS – D and CS – E GAG chains promote axonal growth [122,123]. As noted, expression of CSPGs is greatly upregulated from reactive astrocytes and activated microglia in the CNS after any injury or neurodegenerative disease [124,116, 125]. This enhanced CSPGs accumulates in glial scar and inhibits the regeneration of neurons [126].

CSPGs restrict the growth of neurons by binding to receptors such as Leukocyte common antigen related (LAR), (RPTP σ) and Nogo R (NgR1 and NgR4)[127 128]. LAR and PTP σ activates Rho A and ROCK pathway. Activation of RhoA and ROCK pathway activates LIM kinase inactivating cofilin, negatively affecting the actin dynamics in the growth cone, causing it to retract

and thus restricting the growth of neurons [29, 129,130]. The restricted growth of neurons in the penumbra limits neuroplasticity and functional recovery [116,117].

CSPGs also interact with other ECM molecules to modulate the growth of neurons. CSPGs cause steric hindrance with laminin binding to its integrin receptor [119]. CSPGs also interact with calcium channels on neurons and increase the flow of calcium thus affecting functionality [131]. HSPGs and CSPGs both compete in the binding for RPTP σ. Moreover, CSPGs inhibit oligomerization of RPTP σ, maintaining the activity of the receptor, while HSPGs induce polymerisation and inactivate the RPTP σ activity [95].

CSPGs and OPCs

Oligodendrocyte progenitor cells (OPCs) in the CNS play a vital role in remyelination after injury [73]. The effect of CSPGs on oligodendrocytes is well studied. CSPGs inhibit the growth of OPCs, act as a barrier for the migration of OPCs [132] , and impede the outgrowth of OPCs [133]. Isolated OPCs from mice, when cultured in the presence of CSPGs, showed reduced differentiation to mature oligodendrocytes [132]. Thus, the available data indicates that CSPGs act as a biochemical barrier for the entry of OPCs into glial scar and decrease the differentiation of OPCs to oligodendrocytes thus affecting remyelination activity.

CSPGs and inflammation

CSPGs can interact with microglia releasing various cytokines and growth factors affecting the regeneration process. Microglia reduce phagocytosis in the presence of CSPGs while promoting the release of nitrite and IL-1β [134], while the other studies suggest that CSPGs reduce the release of TNF and nitrates (NO) from microglia stimulated with LPS, thus suggesting CSPGs reduces the pro-inflammatory effect of LPS on microglia [135]. The above-mentioned effect of

CSPGs on microglia were inhibited by blocking the downstream signalling of CSPGs receptor, LAR and PTP σ by intracellular LAR peptide (ILP) and intracellular sigma peptide (ISP) respectively [134] .There are no studies identifying the effect of CSPGs on astrocytes.

CSPGs, specifically with CS – A GAG chain also activate other immune cells including monocytes, inducing the release of IL 1β , CS- B indues B cell proliferation [136]. CSPGs can bind to immunological receptors TLR 2/4/6 on immune cells and activate pro inflammatory cytokine response by activating the nfkB signalling pathway [137]. Further, CS E, CS B and versican bind to P selectin and increase the infiltration of leukocytes[138]. CS E and CS B can bind to CXCL (4, 8, 10 and 12), CCL (2, 5, 8, 11, 20 and 21) and modulate the cellular activity, CS – E also interacts with growth factors like (PTN, midkine, EGF and FGF) and modulate their bioactivity [139].

Thus, available literature suggests CSPGs interact with neurons, OPCs, and microglia in CNS and negatively affect the regeneration of neurons and differentiation of OPCs to mature oligodendrocytes. This again emphasizes that overcoming the inhibitory activity of CSPGs could be a potential target to extend plasticity after brain injury including stroke.

Strategies to overcome inhibitory activity of CSPGs

Modulating neuroplasticity after injury to improve the differentiation and growth of new neurons may lead to improved recovery. One of the approaches to promote the growth of neurons in penumbra involves eliminating the growth inhibitory effect of CSPGs. The inhibitory activity of CSPGs can be overcome by either reducing the synthesis of CSPGs, blocking of downstream signaling, or cleaving the CSPGs.

Inhibitors of GAG chain synthesis

In the CSPG structure, most of the inhibitory activity is driven by GAG chains, which interact with CSPGs receptors. GAG chains attach to the xylose residue on the protein core of the CSPG. Xylosides are analogue of xylose residue, such that compounds such as xyloside like 4 - methylumbelliferyl β-D-xylopyranoside (4-MUX) can inhibit the attachment of the xylose residue to core protein thus inhibit binding of GAG chain to form functional CSPGs [140,141]. In the lysolecithin-induced demyelination mouse model, administration of 4-methylumbelliferyl-β-D-xylopyranoside led to a reduction in CSPG immunoreactivity within the lesion site at the 7-day post-injury mark and demonstrated improved remyelination when assessed at the 21-day post-injury interval [135].

Another compound that can reduce the synthesis of CSPGs is fluorinated glucosamine. Fluorinated glucosamine (fluorosamine) can integrate into CSPGs synthesis process by forming 4 F GlcNAc, which inhibits 4 epimerisation to UDP – GalNAc, thus inhibiting production of a substrate required for CSPGs synthesis [142]. Following demyelination, fluorosamine reduces the expression of CSPGs in the lesion area and increases the concentration of mature oligodendrocytes [97]. In *in vitro* studies, fluorosamine treated astrocytes reduced their expression of CSPGs [143]. When OPCs were grown on a matrix prepared from astrocytes treated with fluorosamine, OPCs showed increased process outgrowth [143]. Thus, fluorosamine can overcome the inhibitory activity of CSPGs by inhibiting its biosynthesis and promoting remyelination after injury.

Inhibitors of CSPG signaling

The inhibitory activity of CSPGs can also be inhibited by blocking downstream signalling from the CSPGs receptors PTP and LAR [144]. CSPGs signals through the RhoA and ROCK pathway; thus, ROCK and RhoA inhibitors can overcome the inhibition of CSPGs. Preclinical

studies with RhoA and ROCK inhibitors show that they promote axon growth, cell survival and improve recovery after stroke, spinal cord injury, or degenerative disease [144]. Another downstream molecule that is involved in CSPGs signaling is GSK – 3b and using lithium (a GSK 3b inhibitor) has shown axonal regeneration an improved recovery in SCI model [145].

Lang *et al* have designed two intracellular peptide that can inhibit the signalling of 2 main CSPGs receptors LAR and PTP σ

1) intracellular sigma peptide (ISP), which targets the intracellular signalling of PTP σ

2) intracellular LAR wedge-domain peptide (ILP), which targets the intracellular signaling of LAR [146]

In a mouse model of MS (Multiple Sclerosis), inhibiting PTP σ signalling via ISP promotes MMP 2 expression, resulting in increased OPC migration and remyelination [147]. In *in vitro* studies with DRG neurons cultured on aggrecan (a common CSPG in the CNS), ISP induces the release of cathepsin B protease that reduces the inhibition of neuronal growth by aggrecan [148]. Additionally, inhibition of PTP and LAR receptors with ISP and ILP can induce differentiation of directly reprogrammed human neural precursor cells (drNPC) to mature neurons in SCI [30]. Thus, blocking the signalling by using either inhibitors or intracellular peptide can overcome the inhibitory activity while keeping the CSPGs in its intact form.

Cleaving of CSPGs

Chondroitinase ABC (ChABC)

The growth inhibitory effect of CSPGs can be overcome using the enzyme ChABC. ChABC is derived from *Proteus vulgaris* [52]. ChABC is an enzyme categorized within the lyases family. It is specialized in catalyzing the eliminative breakdown of polysaccharides that contain (1-4)-β-D-

hexosaminyl, (1-3)-β-D-glucuronosyl, or (1-3)-α-L-iduronosyl linkages, leading to the production of disaccharides that incorporate 4-deoxy-β-D-gluc-4-enuronosyl groups. Thus, ChABC cleaves the GAG side chains from CSPGs and reduces the inhibitory component for neuronal growth.

ChABC has been studied extensively in preclinical models of spinal cord injury, where it promotes the plasticity of sensory neurons and corticospinal tracts [149,150]. Injecting ChABC into the infarct core in a rat model of MCAO increases MAP2 immunoreactivity and reduces GFAP immunoreactivity, suggesting reduced astrocytic activity in penumbra, and was associated with improved functional recovery [151]. Intraspinal injection of ChABC combined with rehabilitative training also improved functional recovery in a photothrombic stroke model in rats [152]. Intracortical ChABC treatment in ipsilesional cortex and rehabilitative training improved functional recovery in another focal stroke model (endothelin 1) for rat [153], and combining ChABC with NEP1-40 (blockade of Nogo -66/NgR signaling) enhances axon regeneration [154]. Thus, there is strong preclinical support for ChABC as a driver of plasticity after injury.

ChABC treatment has also shown to induce migration and promote differentiation of OPCs [155,156]. Several studies have shown that when ChABC was combined with growth factors like EGF, bFGF and PDGF-AA induced NPC survival and differentiation to oligodendrocytes [156,157]. *In vivo* studies of SCI suggest CSPGs negatively regulate OPC growth and remyelination capacity of oligodendrocytes, and upon treatment with ChABC remyelination was restored [158]. ChABC treatment also induces generation of microglia with a pro-repair phenotype, reduced inflammatory profile, and enhanced infiltration of immune cells for improved clearance of debris [159]. ChABC polarizes macrophages to a M2 phenotype, with increased expression of IL 10 and reduced expression of IL 12B [160]. Moreover, some cleaved stubs generated from digestion of CSPGs are known to adopt novel neuroprotective roles [161] 162.162.

ChABC does have several limitations. As ChABC has a bacterial source in *Proteus vulgaris,* there are chances of generating an immunological response. ChBAC also has a short life - time and there needs multiple injections increasing the possibilities of infection [163,164]. Several studies have focused on making ChABC more thermally stable to improve function [163]. More sustainable delivery of ChABC can be achieved by encapsulating ChABC in lipid microtubes which delivered the enzyme for a period of 2 weeks and showed better CSPGs degradation [165]. Another approach to overcome the repeated administration of ChABC is to directly express the enzyme into the animal using viral vectors [166,167]. A novel immune evasive gene therapy was introduced by Burnside *et.al.,* 2018, where the expression of *ChABC* gene was regulated under doxycycline control and can evade T lymphocyte mediated immune response to allow controlled expression of ChABC [168].

Matix Metalloproteinases (MMPs)

An alternative approach to target CSPGs involves endogenous protease families that include the MMPs and ADAMTS (a disintegrin and metalloproteinase with thrombospondin type 1 motifs) [97,169]. These 2 groups of enzymes are known to degrade the core protein in CSPGs and can modify the extracellular matrix affecting the growth of cells [97,170,171]. There are 23 types of MMPs that can cleave different components of ECM. Most of the MMPs are released from the cells as pro MMP form (the inactive form of MMPs) [170].

Cleavage of the prodomain, which is often done other MMPs, leads to active MMPs. Different active MMPs have distinct targets for ECM molecules:

1) MMPs 2, 3, 7, 9, 10, 11, 12, 14, 15, 16, 17, 19, 24, 25, and 26 can cleave fibronectin,
2) MMPs 1, 8, 13, 2, 3, 10, 14, 15 and 16 cleaves collagen.
3) MMPs 1, 8, 13, 18, 2, 9, 3, 7, 14, 15, 16, 24, 12 and 19 cleaves CSPGs and

4) MMPs 8, 13, 2, 9, 3, 7, 14, 12, 9 and 24 cleave laminin [172].

Thus, MMPs can have both beneficial roles in regulating the growth of neurons by eliminating growth inhibitory molecules like fibronectin and CSPGs, while some MMPs can affect growth negatively by eliminating growth promoting molecules like laminin and collagen. MMPs can affect the survival and differentiation of OPCs to oligodendrocytes. MMP 2 and MMP 9 play a major role in migration of neural precursor cells, survival of OPC and migration of OPCs [97,172].

MMP 3 and MMP 13 activate MMP 9 and can also contribute to OPC migration. Oligodendrocytes from MMP 9 null mice showed reduced process outgrowth, and loss of MMP 12 also impairs the differentiation of OPC to oligodendrocytes [173,174]. MMPs including MMP 7 can also cleave transmembrane proteins like N cadherin, which is a cell adhesion molecule and is present on OPCs. This cleavage induces migration of OPCs [173].

Activated microglia and astrocytes release MMPs [169,170]. Microglia activated with LPS induce the release of MMP 9, which is upregulated during an acute phase of spinal cord injury [169]. Inhibition of MMP 3 and MMP 9 from LPS activated microglia reduces the release of pro inflammatory cytokines [175.] During acute ischemic stroke, MMP 2, MMP 3 and MMP 9 are known to disrupt the blood brain barrier (BBB)by degrading tissue junction proteins (claudin, occuldin and ZO 1) [176]

CSPG Modulation: Beyond Digestion

All the above mentioned strategies focus on cleaving the CSPGs or inhibiting the synthesis of CSPGs and blocking the signaling of CSPGs. CSPGs are known to inhibit the regeneration of neurons, migration of OPCs and differentiation of OPCs to oligodendrocytes, all of which are essential for recovery after stroke. However, CSPGs are also associated with beneficial roles. During development, CSPGs play an important role in maintaining structural plasticity and acts as

guiding molecule for neuronal growth [177]. During ischemic stroke, CSPGs are the main component of glial scar and eliminating the synthesis of CSPGs compromises the glial scar integrity and lead to spread of lesion area. Moreover, due to polyanionic nature of CSPGs they can reduce the oxidative stress by scavenging and binding active iron present in local environment around neurons reducing the production of harmful ROS, CSPGs can also buffer the availability of charged ions like sodium, potassium and calcium [178]. Therefore, digesting CSPGs or inhibiting the synthesis of CSPGs can lead to both beneficial and negative effects. Hence, novel therapeutic options that aim to alter the function of CSPGs and limit their involvement in inhibiting neurite growth even in the presence of CSPGs are currently under investigation [33]. One of the potential candidates is the endogenous growth factor PTN.

PTN functions and receptors

PTN is 18 KDa protein and a growth factor important in the development of nervous system that is produced and secreted into the extracellular matrix by neurons and glial cells [48,179]. PTN expression peaks at 12 days and drops at 21 days[180]. During development, PTN is involved in neuritogenesis and synaptogenesis [48,181]. PTN is also known to influence the induction of long-term potentiation (LTP). In *in vitro* study, hippocampal long-term potentiation (LTP) induced by high-frequency stimulation (HFS) is suppressed in the presence of PTN [182]. In *in vivo* studies, PTN knock out mice showed no impairment in memory recognition in Y maze test with 60 min interval intertrial (ITI), while the wild type showed a deficit in memory recognition, thus suggesting role of PTN in memory formation [183]. *In vivo* experiments involving the overexpression of PTN through viral vectors have shown that PTN offers neuroprotection to 6-hydroxydopamine-induced toxicity in tyrosine hydrolase or dopaminergic neurons of striatonigral and nigrostriatal pathways [184].

PTN can interact with various cell surface receptors, including syndecans, nucleolin, protein tyrosine phosphatase receptor type β/ζ (PTPR β/ζ), integrin αVβ3, neuropilin-1and αMβ2 [180]. ALK receptors can be directly activated by binding to PTN or indirectly activated by blocking the activity of PTPRZ [180,185]. Most of the PTN receptors consist of proteoglycans, which are extracellular glycoproteins characterized by the presence of the polysaccharide GAG [180,186]. GAG is a linear polysaccharide comprised of repetitive disaccharide units, with one of the sugars in the disaccharide unit being either glucosamine (GlcN) or galactosamine (GalN), while the other is typically a uronic acid [118]. Thus, by binding to these receptors PTN activates Akt pathway inducing upregulation of growth promoting, differentiation inducing, migration and proliferation [180].

Effect of PTN on Neural stem cells (NSCs)

NSCs are multipotent resident stem cells in the brain. NSCs are generated in the SVZ and SGZ in the brain. Recent studies suggest the beneficial aspects of using NSCs as a treatment regime for stroke [187]. NSCs have been shown to reduce inflammation, provide support to BBB, increase angiogenesis, and lead to generation of new neurons after middle cerebral artery occlusion (MCAO) stroke models in rats and mice [187]. Although NSCs possess all the qualities needed to enhance recovery, the endogenous pool of NSCs is not sufficient to completely restore function, and thus there is a need for exogenous transplantation of NSCs to achieve better recovery. However, there are significant challenges associated with such stem cell therapy, which include immune rejection and tumorogenesis [188]. Signalling of NSCs may be a therapeutic target that avoids these complications. During the entire life span new neurons are generated from NSCs, which reside in SGZ and SVZ regions of the brain [187]. Different factors are released from NSCs that help recovery, including PTN protein. PTN released from hippocampal NSCs provides a survival signal to developing neurons and aids in neurogenesis in adult mice [181]. Additionally,

pericytes are one of the important cells required to form BBB. Ablation of pericytes leads to neuron loss due to BBB break down and intracerbroventricular infusion with PTN prevented neuron loss [189]. Interestingly, neurons become vulnerable to ischemic and excitotoxic injury by silencing expression of PTN from pericytes [190].

Effect of PTN on neurons

After brain injury, there is evidence that expression of PTN is increased in neurons, astrocytes, oligodendrocytes, pericytes and microglia [189, 191, 48]. After injury in CNS and PNS, this increased PTN gene expression have been observed in neurons and glial cells [191,192]. Notably, exogenous administration of PTN induces neurite outgrowth in cultured mice embryonic telencephalic neurons [193]. In a sciatic nerve transection rat model, the denervated Schwann cells showed an increase in PTN gene expression [191]. In a similar model, adult rats were treated with silicone tubes implants containing HEK-293 cells expressing PTN and increased growth of motor neurons was observed, indicating a growth promoting effect of PTN on neurons [191]. In another study where 20 – 40 mm segment of peroneal nerve in rabbit was removed, then treated with hydrogel implants with PTN and GDNF, an increased growth of myelinated neurons was observed [194]. A recent study using a cortical prick injury in mice showed that microinjection of PTN at the site of injury on spinal cord enhanced the growth of neurons at the injured site [195]. Notably, PTN enhances neurite outgrowth by suppressing the growth inhibitory effect of aggrecan (a type of chondroitin sulfate proteoglycans) [195]. PTN also interacts with CSPGs like neurocan and phosphocan in the extracellular matrix [196], which might affect their binding to CSPGs receptors.

Effects of PTN on glial cells

Microglia

According to the limited existing literature, microglia from (PTN-Tg-Thy 1) mice overexpressing PTN in neurons modulate LPS mediated neuroinflammation by enhancing the expression of pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 [197]. In an *in-vitro* experiment, expression of PTN was significantly enhanced 30 min after microglia were subjected to hypoxic OGD condition for 3 h. Notably, PTN regulates the growth of microglia in culture from G1 to S phase by activating ERK 1/2 pathway and blocking the pathway with U0126 (ERK1/2 inhibitor) inhibiting the proliferation of microglia [198]. Further, unpublished data from Dr. Winship's lab has shown that PTN treatment of microglia increases the release of neurotrophic factor, BDNF and decreases the release of pro-inflammatory cytokine IL1β [199]. *In vitro,* microglia treated with PTN showed no significant increase in iNOs or TNF-α but increased the expression of neurotrophic factors like ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), thus promoting anti-inflammatory conditions [179]. With this limited literature, it can be suggested that PTN has both anti-inflammatory and pro-inflammatory effects on microglia, but there is no literature showing the effect of PTN on microglia in the presence of CSPGs. All the findings mentioned above suggest that CSPGs have pro-inflammatory effect on microglia and PTN has an anti-inflammatory effect to some extent, but the effect of PTN in the presence of CSPGs, as would be the case in the CNS, has not been examined [179, 197, 134].

Oligodendrocyte precursor cells (OPCs) and Oligodendrocytes

OPCs express receptor protein tyrosine RPTP β/ζ, which is one of the most prominent receptors for PTN [200]. PTN promotes the proliferation of OPCs by inactivating the signalling of (RPTP β/ζ) [201]. Interestingly, PTN also promotes the differentiation of OPC like OL 1 cells (mouse

oligodendrocyte cell line from p53 deficient mice) cells into myelin expressing oligodendrocyte [202]. In cuprizone model of demyelination, PTPR β/ζ knock out mice showed enhanced remyelination suggesting PTN binds to PTPR β/ζ and inactivates the signalling [203]. PTN treated OL 1 cells in the presence of aggrecan express myelin in differentiating oligodendrocytes [204]. Thus, PTN has the potential to induce proliferation of OPCs in the absence of CSPGs and differentiation of OL 1 cells to oligodendrocytes in the presence of aggrecan.

Astrocytes

Most of the studies done examining the effect of PTN in the CNS focuses on neurons, microglia, and OPCs, and few studies have examined the effect of PTN on astrocytes. Astrocytes being one the major components of glial scar formed after stroke [116], it is of prime importance to understand the effect of PTN on astrocytes in the presence of CSPGs. During brain injury, astrocytes become hypertrophic due to cytokines released from dead and damaged cells, thus forming a glial scar around the damaged tissue to restrict the spread of infection. The limited data available shows the expression of PTP σ (receptor for CSPGs) is increased in activated astrocytes (in the mutant *SOD1* rat model) [205]. The activated astrocytes released CSPGs and the increased expression of PTP σ on these activated astrocytes indicated the potential interaction of CSPGs with PTP σ. The presence of PTN in the glial scar area could potentially interact with astrocytes and alter their activity. LPS stimulation on astrocytes in prefrontal cortex (PFC) of PTN tg mice (overexpression of PTN in Thy 1 positive cells) resulted in less activated astrocytes (as indicated by reduced GFAP expression) compared to control [197]. However, this study does not provide us with the information whether the effect of PTN on astrocytes is due to direct binding of PTN to astrocytes or is due to the effect of cytokines released from microglia or other cells. Therefore, it's important to understand the direct effects of PTN and CSPGs on astrocytes in the glial scar. More studies are required to

determine if PTN can overcome the inhibitory effect of CSPGs on different cell types. Thus, my thesis will focus on looking for potential effects of PTN and CSPGs on neurons and different glial cells and ways to manipulate it in order to increase neuroplasticity after stroke.

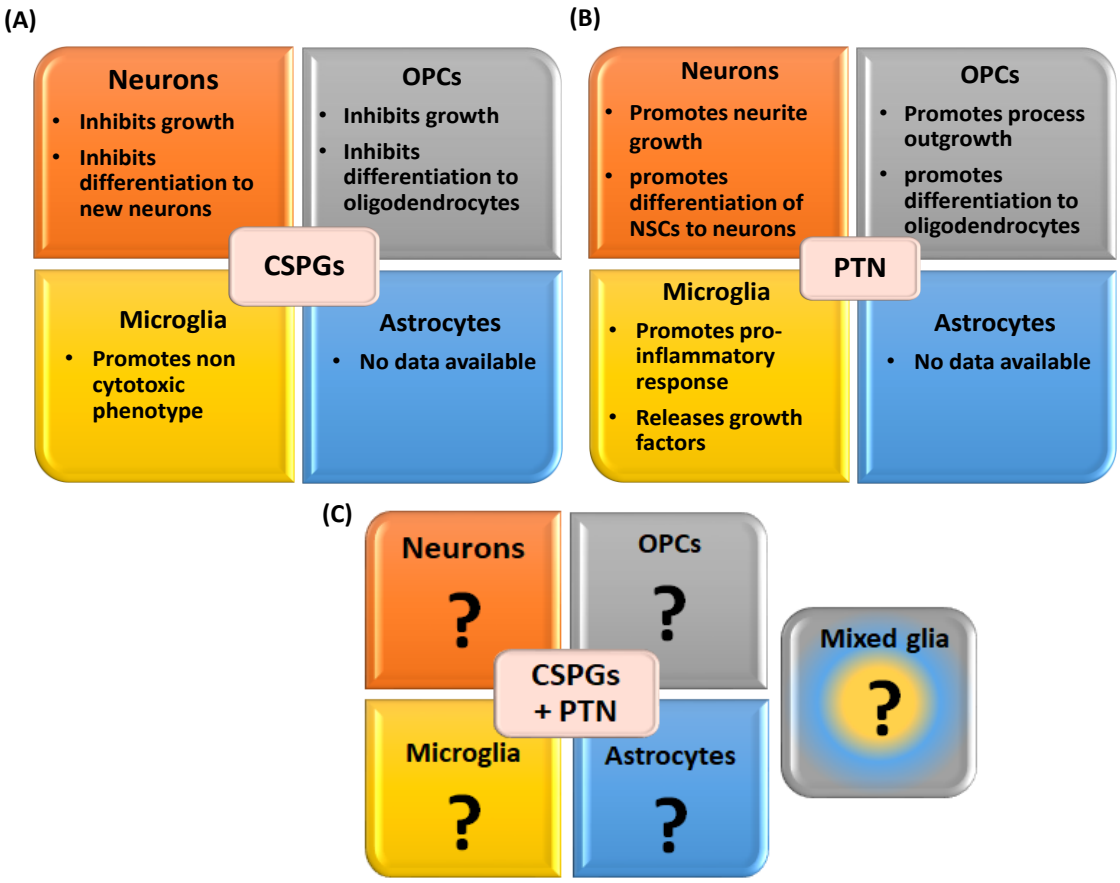


Figure 1.1: Summarized effects of PTN and CSPGs on neurons and glial cells

(A) Summarized effect of CSPGs on neurons and different glial cells (astrocytes, microglia and OPCs) (B) Summarized effect of PTN on neurons and glial cells (astrocytes, microglia and OPCs) (C) Objectives for current thesis to identify the combined effect of PTN and CSPGs on neurons and different glial cells (astrocytes, microglia and OPCs).

Thesis outline and aims

When I started my thesis, the field had identified that CSPGs were a potential target as it inhibits the regeneration process affecting neurons and other glial cells thus delaying recovery after stroke therefore more work was needed to decipher ways to overcome this inhibition. Thus, my dissertation set out to identify the effect of PTN to overcome the CSPGs mediated inhibition.

Chapter 2: Pleiotrophin signals through ALK receptor to enhance growth of neurons in the presence of inhibitory CSPGs

As described above, in CNS injury such as ischemic stroke, the activated glial cells release CSPGs that inhibit the growth of neurons [33], and PTN that promotes the differentiation of NSCs to neurons [206]. PTN promotes neurite outgrowth from neurons cultured on an aggrecan matrix [33]. There are different receptors to which PTN can bind and exert the neurotrophic effect on neurons, which include ALK, syndecan 3 (SDC3), receptor type Z1 (PTPRZ1), and chondroitin sulfate proteoglycan 5 (CSPG5) [48, 180]. Neurons cultured on aggrecan matrix have shown that PTN interacts with glypican – 2 cell surface receptor and promotes the neurite outgrowth [33]. Previous literature has not investigated any dose dependent effect of PTN on neurite outgrowth or identified all key receptors in PTN signalling in the presence of CSPGs. To address this gap, in this chapter we had 2 objectives:

1) To determine the optimum concentration of PTN in inducing neurite outgrowth in the presence of CSPGs
2) To determine the involvement of the ALK receptor in PTN signaling in the presence of CSPGs.

First, we aimed to identify the optimum concentration of PTN at which the neurons show maximum neurite outgrowth. For this, the primary cortical neurons isolated from P0 – P1 rat pups were cultured on the growth inhibitory CSPGs matrix or growth permissive laminin matrix and treated with different concentrations of PTN to determine the impact on neurite outgrowth. This allowed us to identify specific concentrations that can induce maximum neurite outgrowth.

Second, we aimed to investigate the involvement of ALK receptor in PTN signalling. Primary cortical neurons were cultured on growth permissive laminin matrix and growth inhibitory CSPGs matrix and treated with specific concentration of PTN + ALK antagonist, or just with ALK agonist and neurite outgrowth was determined.

Chapter 3: Pleiotrophin modulates the effect of CSPGs on activity of microglia and OPCs

In CNS injury, such as ischemic stroke, activated glial cells (microglia, astrocytes and OPCs) release CSPGs in the glial scar [207]. CSPGs are known to inhibit the growth of OPCs and also inhibit the differentiation to oligodendrocytes [133]. CSPGs are also known to inhibit the regeneration of neurons, thus CSPGs are a limiting factor in the recovery process after stroke.

PTN is known to induce the differentiation of OPC to oligodendrocytes [208]. Similar to the upregulation of CSPGs abundance, activated glial cells also show an upregulation in the expression of PTN [209]. All the available data identified independent effect of CSPGs or PTN on differentiation of OPCs but there are no studies identifying the dose – dependent effect of PTN on the differentiation of OPCs in the presence of CSPGs. Therefore, to consider PTN as a therapeutic protein to enhance recovery after stroke, we hypothesize that PTN will induce the differentiation of OPC to oligodendrocytes, even in the presence of CSPGs. To test this hypothesis, isolated OPCs were cultured on growth permissive laminin matrix and growth inhibitory CSPGs matrix and

treated with different concentrations of PTN, the total number of completely differentiated and differentiating cells were counted and their growth profile was determined for the effect of PTN.

This chapter also examined the effect of PTN on microglia. In CNS injury, activated microglia release CSPGs and PTN[209]. CSPGs induce a noncytotoxic phenotype in microglia by reducing the expression of pro inflammatory cytokine in response to LPS [135], while PTN promoted the release of pro inflammatory cytokine from LPS-stimulated microglia [210]. All the available literature previously recognized the independent effects of PTN or CSPGs on both unstimulated microglia and LPS-stimulated microglia. However, in the context of any CNS injury, infiltrating macrophages and T cells release IFN γ, subsequently activating microglia [35]. Hence, to consider therapeutic potential of PTN for stroke it becomes important to investigate the synergistic impact of PTN and CSPGs on unstimulated microglia and IFN γ stimulated microglia. To address this gap, the isolated microglia were cultured on Poly L Lysine matrix or on CSPGs matrix and treated with different concentrations of PTN, the media was collected and assayed for pro inflammatory cytokines released along with some other parameters to assess the effect of PTN on microglia in the presence of CSPGs.

Chapter 4: Pleiotrophin modulates the effect of CSPGs on the activity of astrocytes and mixed glia

Astrocytes play a major role in maintaining normal physiological function of neurons by buffering excess neurotransmitters or K^+ from the extracellular space [211,212]. In CNS injury, activated astrocytes play a major role in forming glial scar to restrict the spread of infarction to other parts of brain. Activated astrocytes release CSPGs, which are a major component of glial scar [209,213]. All the available data identifies the effect of CSPGs or PTN on different glial cells,

including microglia and OPCs, but there are no studies that focuses on identifying the effect of CSPGs or PTN together on astrocytes.

To address this gap of knowledge one of the objectives of this chapter focused on identifying the effect of PTN on astrocytes in the presence of CSPGs. For this, isolated astrocytes were cultured on poly L Lysine (PLL) matrix or CSPGs matrix and treated with different concentrations of PTN, the media was assessed for pro inflammatory cytokine released. To further probe cell-cell interactions, we expanded our study to determine the effect of PTN on mixed glial cell population that consists of astrocytes, OPCs and microglia. We cultured the mixed glial cells on PLL or CSPGs matrix and treated with different concentrations of PTN with or without secondary inflammatory stimulus (IFN γ) and assayed for different pro inflammatory cytokines.

Chapter 5: The overall goal of my thesis is to identify the potential effect of PTN in overcoming the inhibitory activity of CSPGs on neurons and different glial cells (astrocytes, microglia, OPCs and mixed glia). In a closing chapter I will summarize the most pressing experiments that are needed to work towards the utility of PTN as therapeutic target molecule to promote recovery after stroke.

Chapter 2: Pleiotrophin signals through ALK receptor to enhance growth of neurons in the presence of inhibitory CSPGs

A version of this chapter appears as a published manuscript in Neural Insights (Gupta SJ, Churchward MA, Todd KG, Winship IR. Pleiotrophin Signals Through ALK Receptor to Enhance the Growth of Neurons in the Presence of Inhibitory Chondroitin Sulfate Proteoglycans. Neurosci Insights. 2023 Jul 15;18:26331055231186993. doi: 10.1177/26331055231186993. PMID: 37465214; PMCID: PMC10350765.

Abstract

Chondroitin sulfate proteoglycans (CSPGs), one of the major extracellular matrix components of the glial scar that surrounds central nervous system (CNS) injuries, are known to inhibit the regeneration of neurons. This study investigated whether pleiotrophin (PTN), a growth factor upregulated during early CNS development, can overcome the inhibition mediated by CSPGs and promote the neurite outgrowth of neurons *in vitro.* The data showed that a CSPG matrix inhibited the outgrowth of neurites in primary cortical neuron cultures compared to a control matrix. PTN elicited a dose dependent increase in the neurite outgrowth even in the presence of the growth inhibitory CSPG matrix, with optimal growth at 15 ng mL^{-1} of PTN (114.8% of neuronal outgrowth relative to laminin control). The growth promoting effect of PTN was blocked by inhibition of the receptor anaplastic lymphoma kinase (ALK) by alectinib in a dose dependent manner. Neurite outgrowth in the presence of this CSPG matrix was induced by activation of the protein kinase B (AKT) pathway, a key downstream mediator of ALK activation. This study identified PTN as a dose-dependent regulator of neurite outgrowth in primary cortical neurons cultured in the presence of a CSPG matrix, and identified ALK activation as a key driver of PTN-induced growth.

Significance statement

Function in the central nervous system (CNS) is attributed to the complex interactions of neurons and glia. These cells are anchored in extracellular matrix (ECM) which constitutes about 10% - 20% of brain volume. Cells in the brain produce different components of the ECM in brain including chondroitin sulfate proteoglycans (CSPGs). After a nervous system injury, glial cells produce excess CSPGs that restrict the regeneration of neurons, thus limiting functional recovery. This study examines the role of the endogenous growth factor pleiotrophin (PTN) in driving the

growth of neurons even in the presence of inhibitory CSPGs, and anaplastic lymphoma kinase (ALK) receptor as a key mediator by which PTN potentiates growth.

Introduction

Chondroitin sulfate proteoglycans (CSPGs) are a major component of the extracellular matrix that surrounds cells of the central nervous system (CNS) [50]. CSPGs maintain CNS health by regulating growth of axons during development and protecting against oxidative stress [214]. After CNS injury, activated glial cells increase synthesis of CSPGs to form a glial scar that contains the injury site [50,215]. The scar reduces lesion growth but also acts as a barrier for the regeneration of neurons that may limit functional recovery [50,216,217]. CSPGs are also upregulated in neurodegenerative conditions including Alzheimer's disease (AD), Pick bodies in Pick's disease, and Lewy bodies in Parkinson's disease (PD) [218]. Several experimental approaches have been investigated to neutralize the growth inhibitory effect of CSPGs *in vivo*, including digestion of CSPGs by chondroitinase ABC (ChABC), knockdown of CSPG polymerisation enzymes by RNA interference (RNAi), and peptide blocking the signalling of two major receptors of CSPGs: protein tyrosine phosphatase sigma (PTPσ) and leukocyte antigen receptor (LAR) [219]. Although these strategies have shown positive effects on the growth of neurons in model systems, there are disadvantages that may limit translation to clinical use. Both ChABC and siRNA are exogenous macromolecules that must be delivered directly to the injury site, have short half-lives *in vivo*, and could induce immunological response due to repeated administration [220]. Potentiating regeneration of neurons without degrading the glial scar may be possible via the heparin-binding growth factor pleiotrophin (PTN). PTN has long half life [195, 221] and may overcome the disadvantage of repeated administration associated with the use of ChABC and siRNA. PTN is a developmentally regulated protein whose expression peaks (in rats) 3-4 weeks after birth [222]. Notably, PTN expression is low

in adults but increases transiently after CNS injury [222]. PTN is associated with neuroplasticity including the maturation of new neurons and induction of neurite outgrowth [223]. Phosphocan is a major type of CSPGs present in the brain matrix whose structure resembles the extracellular component of PTPRζ (one of the receptors for PTN) [50,218]. *In vitro* studies with spiral ganglion neurons have shown that PTN induces the neurite outgrowth in spiral ganglion [224] and in primary cortical neuronal cultures the growth promoting activity of PTN was suppressed by anti phoshocan antibody, thus suggesting the signalling happens via PTPRζ [225]. Notably, PTN may reverse aggrecan (a prominent CSPG) mediated inhibition *in vitro* by preventing the binding of aggrecan to PTP σ [195]. In Parkinson's disease (PD), PTN has shown to reduce nigrostriatal degeneration and improve functional recovery [218]. Notably, PTN has a sustained presence in the tissue when injected into the nervous system directly. Moreover, identifying key receptors involved in PTN-induced neuroplasticity may allow for systemic pharmacotherapy.

PTN has several putative cell surface receptors, including receptor protein tyrosine phosphatase ζ (RPTPζ/PTPRZ), syndecans, nucleolin, neuropilin-1, integrin αVβ3 and αMβ2, N-syndecan receptor, glypican 2, neuroglycan-C, and anaplastic lymphoma kinase (ALK)[195,222]. Moreover, PTN can integrate into the extracellular matrix, limiting CSPG interactions with growth-inhibitory receptor PTPσ for a sustained period [195].

This study investigates the potential role of ALK receptor activation by PTN to increase the growth of neurons in the presence of a CSPG matrix. ALK is expressed during early development in CNS, with low expression in the adult central nervous system [226,227] ALK signalling is critical for differentiation of neuronal progenitor cells to neurons and regulates their survival [228, 223]. By binding to CSPGs, PTN also reduces phosphatase activity of RPTP ζ, which increases activity of ALK [222]. Moreover, in developing neurons PTN signals directly via ALK

receptor binding [223,193] and in SK-N-SH cells PTN promotes cell and neurite outgrowth [229]. Thus, the evidence suggests that the ALK receptor may facilitate neuroplasticity even in the presence of CSPGs. Here, we directly investigated *in vitro* the role of PTN signaling via ALK in driving neuron growth in the presence of inhibitory CSPGs. Our data reveals a dose dependent effect of PTN on neurite outgrowth in the presence of CSPGs. Notably, selective pharmacological inhibition of the ALK receptor attenuated the growth promoting effect of PTN.

Materials and Methods

Matrices preparation:

Coverslips were coated with 100 µg mL^{-1} Poly-L-Lysine (Sigma-aldrich, P5899, USA) for 2 hours. After 3 washes with water, the coverslips were coated with either a growth permissive matrix -10 µg mL^{-1} of laminin (Corning, 354232) or an inhibitory matrix - 10 µg mL^{-1} laminin + 1.25 µg mL^{-1} of CSPGs (Sigma aldrich, CC117) for 2 hour and washed 2 times with PBS.

Primary cortical neuronal culture:

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. Rat primary cortical neurons were isolated from 0-1day old Sprague Dawley rat pups. The cortices were dissected and digested with TrypLE (Gibco, 12605-028) for 15 minutes at 37°C. The cells were dissociated from tissue by trituration in neurobasal A medium (Thermofisher, 1088802) containing B27 supplement (1:50 v/v) (Gibco, 17504-044), antibiotics and GlutaMAX (Gibco, 35050-061). The cell suspension was seeded at 20,000 cells / well on coverslips with different matrices and incubated at 37 °C, 5% CO_2 for 1 hour. Following incubation, media was replaced with media containing different concentration of recombinant

human PTN (rhPTN, R&D systems 252 - PL) or the drugs alectinib (Toronto Research Chemicals, C183665) or SC79 (Selleck Chemicals, S7863) and cells were incubated for 72 h.

Microscopy, fluorescent immunostaining and image analysis

Cells were fixed after 72 h of treatment with 5% formaldehyde solution for 15 min. and washed with PBS. The fixed samples were blocked with 10% normal horse serum in PBS containing 0.1% Tritton-X 100 and washed with PBS. Samples were stained overnight with microtubule-associated protein 2 (MAP 2) antibody (1:500, Sigma aldrich M9942), ALK (1:500, abcam ab190934) rinse with PBS. The primary antibody MAP 2 was detected with secondary antibody donkey anti-mouse Alexa 647 (1:500 abcam ab150107) and ALK was detected with donkey anti-rabbit Alexa 488 (1:500 abcam ab150073), nucleus was stained with Hoechst 33342 (1:1000, Invitrogen, 62249). The images were acquired using Leica DMI6000B fluorescent microscope with Leica DFC365FX camera at objective magnification of 20X lens with 0.7 numerical aperture. The images were analysed using Simple Neurite Tracer (SNT) plugin in Fiji [230]. All the neurite outgrowth from soma of each neuron was traced and any neurite not originating from soma was considered as branched neurite. Total neurite outgrowth, branched neurite outgrowth and number of neurons possessing neurite outgrowth for neurons in each treatment conditions were manually traced and counted using SNT plugin in Fiji. The data was then normalized to respective laminin control.

Statistics

Statistical analyses of data from fluorescent images were carried out using one-way ANOVA followed by Dunnett's multiple comparisons test for significance between treatment groups. n represents a single independent experiment (i.e. an independent culture preparation) with a

minimum of three technical replicates. Each technical replicate represents a well in a 24 well culture plate. All statistical analyses were done using Graphpad Prism version 9.1.2.

Results

Pleiotrophin induces neurite outgrowth in the presence of CSPGs

CSPGs are known to inhibit the growth of neurons. In order to understand the effect of PTN on neurite outgrowth, cortical neurons were cultured on the growth permissive (laminin) matrix or on the growth inhibitory (laminin + CSPGs) matrix and treated with different concentrations of PTN (5 ng mL^{-1} – 20 ng mL^{-1}) for 72 h. MAP2 immunofluorescence was used to quantify neurite outgrowth. Neurons showed extensive neurite outgrowth on the laminin matrix (Fig. 2.1 A – C) that was inhibited by CSPGs (Fig. 2.1 D – F). PTN restored neurite extension in a dose dependent manner (Fig. 2.1 G – I). Neuronal morphology was analysed using the Simple Neurite Tracker plugin for ImageJ [231] (Fig. 2.1 J). A dose dependent effect of PTN on total neurite outgrowth was observed, with PTN counteracting the inhibitory effect of CSPGs at concentrations below 20 ng mL^{-1} and maximal effect at 15 ng mL^{-1} concentration (Fig. 2.1 K). PTN treatment increased the complexity of cortical neuron neurite growth by increasing branched neurite growth, with maximum branch length observed at 15 ng mL^{-1} PTN (Fig. 2.1 L), and increased the number of neurons with neurite outgrowth at 10-15 ng mL^{-1} PTN (Fig. 2.1 M). This data suggests that PTN at specific concentrations potentiates neurite extension from cortical neurons grown in CSPGs matrices.

Pleiotrophin signals via ALK receptor on neurons

PTN signals by inactivating the phosphatase activity of PTPRζ [232]. Currently available pharmacological inhibitors of PTPRζ are quite limited [218]. Thus, identifying other potential

receptors for PTN may identify new targets to drive neuron growth. ALK is a putative PTN receptor, and is broadly expressed on cortical neurons *in vitro* (Fig. 2.2 A – C). Treatment of primary neuronal cultures with alectinib, which selectively inhibits phosphorylation of ALK and therefore blocks activity of ALK [233,234,235], was used to probe the involvement of the ALK receptor in PTN signalling. Alectinib inhibited neurite outgrowth from PTN treated neurons cultured on a CSPGs matrix (Fig. 2.2 D – O), exhibiting a dose dependent effect on neurite outgrowth (Fig. 2.2 P), and on neurite branching (Fig 2.2 Q). Alectinib also reduced the number of neurons possessing neurite outgrowth in the presence of PTN (Fig. 2.2 R), suggesting that PTN signaling via ALK receptors is key to its growth inducing effects in the presence of CSPGs.

Activating the AKT pathway drives neurite growth

The protein kinase B (AKT) pathway is a key downstream transducer of PTN/ALK signalling [223]. To investigate the involvement of this pathway in the regeneration of neurons, cortical neurons cultured on a CSPG matrix were treated with the AKT activating compound SC79 in the absence of PTN [223]. SC79 treated neurons showed a dose dependent enhanced neurite outgrowth even in the presence of CSPGs (Fig. 2.3 A – N)

Discussion

This study examined the effect of PTN on the growth of cultured cortical neurons in the presence of CSPGs. Neurite outgrowth of primary cortical neurons in the presence of CSPGs was enhanced by PTN treatment in a dose dependent manner, with PTN counteracting the inhibitory effect of CSPGs at concentrations below 20 ng mL^{-1}. These findings support PTN as a candidate to restore neurite extension in CSPG - rich lesion areas after CNS injury and other neurological conditions. This is consistent with cell growth induced by PTN in studies of neurodegenerative disease. PTN has been shown to promote cell survival signal in dopaminergic neurons invitro and

in a mouse model of Parkinson's disease PTN was shown to promote survival of grafted dopaminergic neurons, thus improving functional recovery of the nigrostriatal pathway [218,236]

The available literature indicates the involvement of ALK in neuron like differentiation of PC12 cells [237] and depletion of ALK receptor attenuates neuronal proliferation and neurogenesis [238]. Here, attenuation of neurite growth due to alectinib treatment provides direct evidence for the necessity of ALK activity in neurite extension, identifying a further avenue for investigation for development of ALK agonists to drive neuroplasticity.

Activating AKT signalling with SC79 induced more moderate neuronal growth relative to to PTN treatment, with 15 ng mL^{-1} PTN showing 114.8 % as compared to 5 μM SC79 with 78.43% increased neurite outgrowth compared to laminin control. The reason for reduced effectiveness of SC79 relative to PTN incubation could reflect other potential downstream activators of ALK signalling. ALK is known to activate many pathways including phospholipase C γ, Janus kinase (JAK), PI3K-AKT, mTOR and MAPK signaling cascades[232], [237]. Thus, PTN may activate multiple growth associated pathways via ALK, and blocking ALK activity with alectinib inhibits multiple pathways beyond AKT that may contribute to the significant reduction in neurite growth even at the lowest tested concentrations (Figure 2-8).

Notably, PTN may also modulate the activity of glial cells including OPCs (oligodendrocyte progenitor cell) and microglia. PTN induces differentiation of OPCs to mature oligodendrocytes, thus promoting myelination of developing neurons [232], and could therefore potentiate remyelination after injury. Microglia increase their release of neurotrophic factors including ciliary neurotrophic factor (CNTF), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) after stimulation with PTN [239]. Thus, while the data presented here demonstrate direct actions on neurons via ALK, PTN is a strong candidate to generate an

environment favouring the neuronal growth and its functionality by actions on multiple cell types. These data suggest PTN signaling may be an exciting approach to enhancing neuroplasticity after CNS injury, though the dose-response relationship for PTN in the presence of CSPGs will be important to verify *in vivo*. PTN/ALK signalling may therefore be a potential target to induce neuron regeneration after CNS injury or degenerative diseases associated with CSPG upregulation, including Alzheimer's, Parkinson's, and multiple sclerosis.

Figures

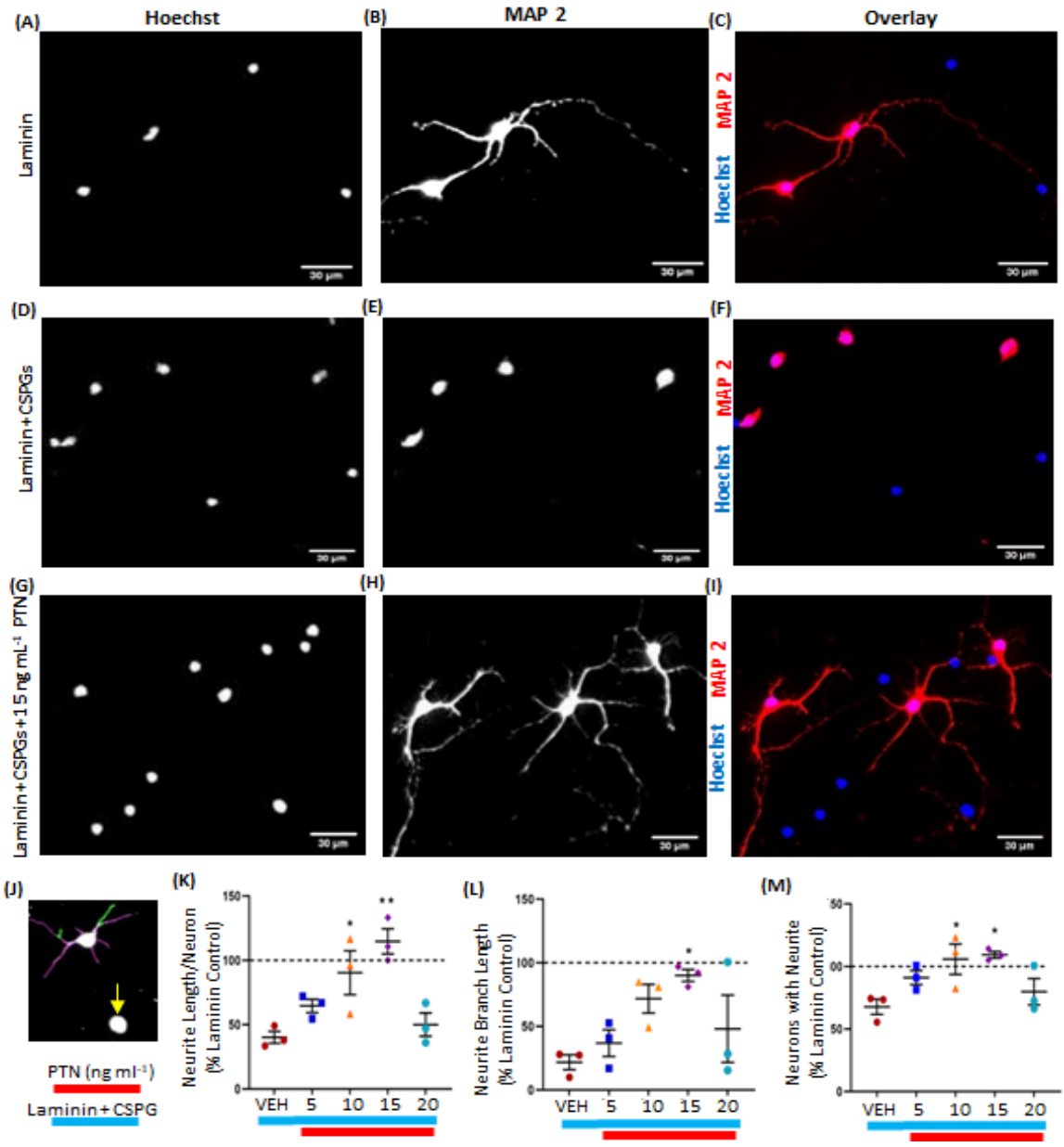


Figure 2.1: Effect of PTN on the neurite outgrowth in the presence of CSPGs

All the images were acquired at 20 X magnification. Neurons cultured on laminin (growth permissive matrix) and stained for **(A)** Hoechst, **(B)** MAP 2 (Microtubule associated protein 2) with Alexa fluor 647, **(C)** overlay. Neurons cultured on laminin + CSPGs (growth inhibitory matrix) and stained for **(D)** Hoechst, **(E)** MAP 2 with Alexa fluor 647 **(F)** overlay. Scale bar: 30

μm. Neurons cultured laminin + CSPGs (L + C, growth inhibitory matrix) and treated with 15 ng mL^{-1} pleiotrophin (PTN) and stained for **(G)** Hoechst **(H)** MAP 2 with alexa fluor 647 **(I)** overlay. Scale bar: 30 μm. **(J)** Neurite outgrowth traced using simple neurite tracer plugin (SNT) in Fiji, total neurite growth (highlighted in purple + green), branched neurite outgrowth (highlighted in green) and neurons with no neurite outgrowth (indicated by yellow arrow). **(K)** PTN induces neurite growth in the presence of CSPGs (ANOVA, $F_{(4,10)}$=9.028, P=0.0024). **(L)** PTN induced branched neurite growth in the presence of CSPGs (ANOVA, $F_{(4,10)}$=3.740, P=0.0413) **(M)** PTN increases number of neurons with neurite growth in the presence of CSPGs (ANOVA, $F_{(4,10)}$=4.560, P=0.0235). **(K – M)** Error bars represent standard error of mean (SEM), symbols *, ** represent $p<0.05$ and 0.01, respectively, on Dunnett's multiple comparisons against control. All data are based on 3 independent experiments with a minimum of three technical replicates.

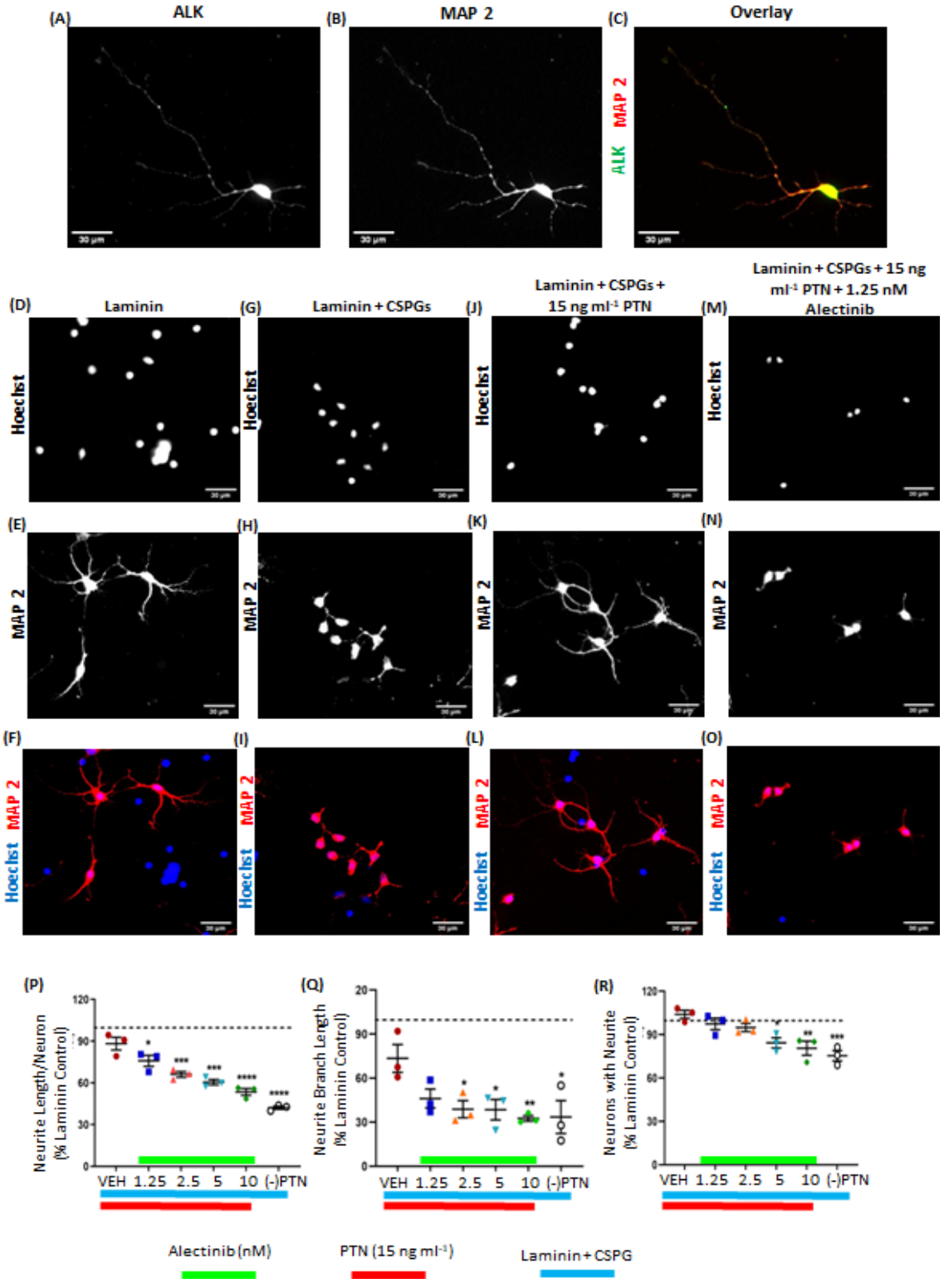


Figure 2.2: Effect of alectinib on the neurite outgrowth in the presence of CSPGs

All the images were acquired at 20 X magnification. Expression of ALK in cortical neuronal culture and stained for **(A)** ALK with Alexa fluor 488 **(B)** MAP 2 with Alexa fluor 647 and **(C)** overlay. Scale bar: 30 µm. Based on PTN dose response data from figure 2.1 (K, L, M) 15 ng mL^{-1} of PTN concentration showed maximum growth response therefore 15 ng mL^{-1} PTN was used for alectinib experiments. Cortical neurons cultured on laminin matrix **(D – F)**. Cortical neurons cultured on Laminin + CSPGs matrix **(G- I)**. Cortical neurons cultured on Laminin + CSPGs matrix and treated with 15 ng mL^{-1} PTN **(J – L)**. Cortical neurons cultured on Laminin + CSPGs matrix and treated with 15 ng mL^{-1} PTN with 1.25 nM alectinib **(M – O)**. After 72 h incubation neurons were stained for Hoechst (D,G,J,M) and MAP2 (E,H,K,N) with Alexa fluor 647. Scale bar: 30 µm. **(P)** Neurite growth induced by PTN in the presence of CSPGs is blocked by alectinib (ANOVA, $F_{(5,12)}$=30.48, P<0.0001). **(Q)** PTN induced branched neurite growth in the presence of CSPGs is blocked by alectinib (ANOVA, $F_{(5,12)}$=4.078, P=0.0214). **(R)** Alectinib reduced number of neurons with neurite growth in the presence of PTN in the presence of CSPGs (ANOVA, $F_{(5,12)}$=9.068, P=0.0009). **(P – R)** Error bars represent standard error of mean (SEM), symbols *, ** and ***/**** represent P<0.05, 0.01, and 0.001 respectively. All data are based on 3 independent experiments with a minimum of three technical replicates.

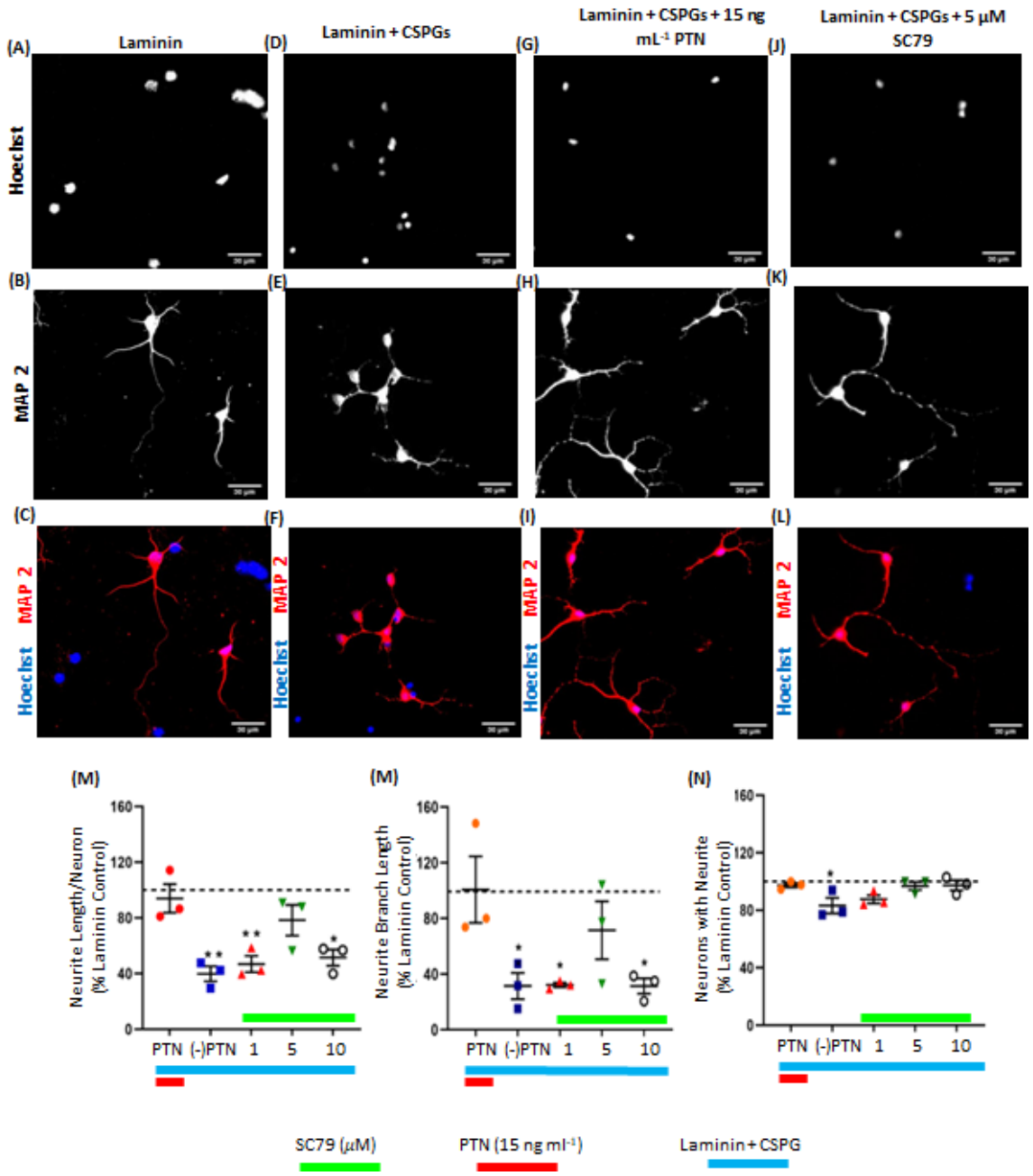


Figure 2.3: Effect of SC79 on the neurite outgrowth in the presence of CSPGs

All the images were acquired at 20 X magnification. Cortical neurons cultured on **(A – C)** Laminin matrix, **(D- F)** Laminin + CSPGs (L + C) matrix, **(G – I)** Laminin + CSPGs matrix and treated with 15 ng mL^{-1} PTN, **(J – L)** Laminin + CSPGs matrix and treated with different concentration of SC79 (AKT activator). After 72 h incubation neurons were stained for stained MAP2 with Alexa

fluor 647 (B,E,H,K) and Hoechst (A,D,G,J). Scale bar: 30 µm. **(M)** SC79 induced neurite growth in the presence of CSPGs (ANOVA, $F_{(4,10)}$=8.222, P=0.0033). **(N)** SC79 increases branched neurite growth of neurons in the presence of CSPGs (ANOVA, $F_{(4,10)}$=4.441, P=0.0254). **(O)** SC79 increases number of neurons with neurite growth in the presence of CSPGs (ANOVA, $F_{(4,10)}$=3.699, P=0.0425). **(M – O)** Error bars represent standard error of mean (SEM), symbols * and ** represent p<0.05 and 0.01, respectively, on Dunnett's multiple comparisons against vehicle control. All data are based on 3 independent experiments with a minimum of three technical replicates.

Chapter 3 : Pleiotrophin modulates the effect of CSPGs on activity of microglia and OPCs.

Introduction

The myelination of neurons is essential for efficient signal transduction of nerve impulses in neurons [240, 241]. Oligodendrocytes are the glial cells responsible for forming the myelin sheath around neuronal axons in the CNS. Oligodendrocytes are generated by differentiation of oligodendrocyte progenitor cells (OPCs). OPCs constitute about 7 – 9 % of cells in white matter and 2 – 3 % in gray matter [242]. Generation of oligodendrocytes is guided by various external factors that govern the proliferation of OPCs or differentiation of OPCs to oligodendrocytes [243]. Notably, neurons play a significant role in dictating the proliferation and differentiation of OPCs to mature oligodendrocytes [76,81].

Effect of neuronal activity on OPCs and oligodendrocytes

Neurons release various growth factors, including PDGF AA, and neurotransmitters that can affect the proliferation of OPCs and differentiation of OPCs to myelinating oligodendrocytes [244] Additionally, OPCs have receptors neurotransmitters for neurotransmitters including glutamate and GABA[244]. Moreover, [245] neuronal activity plays a significant role in regulating the survival of premyelinating oligodendrocytes and myelinating oligodendrocytes[246]. For example, intraocular injections with Tetrodotoxin (TTX) reduces the proliferation of OPCs in rat optic nerve [247]. .

OPCs in injury

Oligodendrocytes are sensitive to oxidative stress, inflammatory conditions, and excitotoxicity resulting from ischemic stroke [248], with the lack of oxygen in the periinfarct area surrounding the core inducing death of oligodendrocytes cells and demyelination [249]. After an injury, the OPCs migrate to the damaged site to differentiate into mature oligodendrocytes [248]. This remyelination process is inefficient and fails to effectively myelinate all the developing or

regenerating neurons as the OPCs become stalled during differentiation [244]. Several factors are responsible for the failure of differentiation of OPCs, including insufficient access to growth and differentiation factors and upregulated components of the extracellular including CSPGs and HSPGs [243, 249, 250].

Effect of CSPGs on OPCs and oligodendrocytes

CSPGs are proteoglycans consisting of protein core and GAG side chains [116]. CSPGs are an extracellular matrix component that serves as guidance cues during CNS development [251, 52]. During an injury, activated glial cells release CSPGs, which account for one of the major components of the glial scar [252, 253]. This glial scar serves as a barrier to restrict the spread of damage to other healthy parts of the brain [50, 254]. The CSPG component of the glial scar is essential to maintain its integrity, but CSPGs are also known to inhibit the migration of OPCs and their differentiation to mature oligodendrocytes, affecting the remyelination process [158, 255].

Several approaches have been identified to overcome the inhibitory effect of CSPGs by inhibiting the synthesis of CSPGs, cleaving of CSPGs using chondroitinase ABC (ChABC) or by silencing the signalling of 2 major CSPG receptors, PTP σ and LAR, by using siRNA [158, 256, 257, 220]. These strategies can have positive effects for treating neuronal injuries, but also have limitations. ChABC and siRNA have a short half-life and thus need to be administered frequently, which increases the chances of infection or complication. Moreover, ChABC is from a microbial source which can be immunogenic [220]. The treatment of siRNA and ChABC cannot specifically eliminate CSPGS in glial scar only. On the contrary, this treatment can eliminate the CSPGs in the surrounding matrix and thus can affect the functioning of neurons surrounded by perineuronal nets [258]. Chemicals like xyloside or fluorinated glucosamine disaccharide that can reduce production of CSPGs are also not specific to CSPGs in the glial scar and can affect the synthesis of CSPGs

beyond the glial scar [258]. Therefore, identifying methods that can potentiate the migration, proliferation and differentiation of OPCs to mature oligodendrocytes, even in the presence of CSPGs, holds a great potential to expedite the process of regeneration after stroke.

Effect of MMPs on OPCs, oligodendrocytes and microglia

The OPC-dependent remyelination process can also be modulated by manipulating the ECM [259]. Matrix metalloproteinases (MMPs) are expressed in very low to undetectable concentrations under normal physiological concentration, and the expression increases during an injury or disease [260]. As noted, CSPGs inhibit the remyelination process by reducing the differentiation and migration of OPCs [261, 158]. MMPs can cleave various ECM components, including CSPGs, fibronectin, NG2, CSPGs, laminin, collagen and tenascin C [262]. Fibronectin inhibits the formation of branched process from oligodendrocytes in the absence of MMP 9 [263]. MMP 2 can cleave various components of ECM (integrin, fibronectin, CSPGs and vitronectin) thus facilitates the migration of OPCs, differentiation and its survival [264]. Similarly, MMP 9 can cleave fibronectin, NG2 and vitronectin thus favouring OPC migration, oligodendrocyte process extension [264] Thus, inhibiting MMP 2 and MMP 9 activity decreased survival and growth of OPCs and oligodendrocytes [260].

Microglia cultured on CSPG matrices *in vitro* have increased the expression of MMP 9 and MMP 2 [265]. Activated microglia are a major source of MMP expression during an injury. Inhibiting the activity of MMP-9 prevents the cleavage of CSPGs and maintains the integrity of the glial scar, thereby reducing the spread of infarction. This suggests that CSPGs are essential for the formation of the glial scar and for restricting the spread of infarction [266]. Overall, the available literature suggests that reduced expression or activity of MMP 9 and MMP 2 could be beneficial during the early stages of injury or disease to restrict the spread of infarct, while during the later stages of

injury when the glial scar is formed, restoring the activity of MMPs is essential to promote the OPC differentiation.

Effect of PTN on OPCs

Another approach to promote differentiation of OPCs is to neutralize the inhibitory activity of CSPGs without cleaving or inhibiting the synthesis of CSPGs. Mouse oligodendrocyte cell lines from p53 deficient mice (OL1) cells, when treated with protamine (a heparin antagonist), stimulated differentiation in the presence of aggrecan (a type of CSPG) [241]. Another protein that potentiates the differentiation of oligodendrocytes is pleiotrophin (PTN). PTN is developmentally regulated protein whose expression peaks around 12 days post-natal, and gradually drops by day 21 [180]. OPCs in CNS expresses the receptor protein tyrosine RPTP β/ζ, which is a potent receptor of PTN[201]. PTN promoted differentiation of OPC like OL 1 cells [267] and also potentiates the proliferation of OPCs by inactivating Protein tyrosine phosphatase receptor type Z (PTPRZ) [268]. Thus, PTN has the potential to induce proliferation and differentiation of OPC. We hypothesised that PTN can induce differentiation of OPC to oligodendrocytes in the presence of CSPGs.

To test this hypothesis, in this chapter OPCs were isolated from mixed glia culture prepared from 2-day-old CX3CR1/GFP mice pups. OPCs were cultured on a CSPGs matrix and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM) and various markers for oligodenrocyte differentiation lineage were quantified to determine the differentiation of OPCs to oligodendrocytes.

Effect of CSPGs on microglia

Inflammation also plays a major role in determining the fate of differentiation of OPCs to mature oligodendrocytes, creating an environment favouring neuroplasticity after an injury.

Microglia are the primary innate immune cells in the CNS, which get activated during an injury or neurological disease, releasing CSPGs and pro-inflammatory cytokines [117, 248]. During the early stage of injury, the microglia migrate to the injury site and release proinflammatory cytokines to activate astrocytes to form glial scar [248]. Microglia-mediated tumor necrosis factor (TNF) release is required for phagocytic signalling necessary for clearance of myelin debris and generation of new oligodendrocytes [269]. Microglia also interact with CSPGs, and *in vitro* data suggests reduced release of proinflammatory cytokine TNF and nitric oxide (NO) in response to treatment with LPS in the presence of CSPGs, suggesting that CSPGs reduce the pro-inflammatory activity of microglia [265] During the early stages of injury, some inflammatory activity of microglia is essential for clearing up debris. Therefore, augmenting inflammatory activity in microglia in the presence of CSPGs early after stroke might promote the recovery process.

Effect of PTN on microglia

PTN also impacts the activity of microglia, causing the release of neurotrophic factors [239]. PTN promotes the release of pro-inflammatory cytokines (TNF, IL 6 and MCP – 1) from LPS-stimulated microglia [270]. Thus, PTN induces a pro-inflammatory effect, while CSPGs suppress the pro-inflammatory effect on LPS-stimulated microglia [270, 265]. In summary, the available data indicates that under the influence of CSPGs, stimulated microglia reduced the release of pro inflammatory cytokines while PTN potentiated the release of pro inflammatory cytokines. In *in vivo* conditions, microglia present in glia scar are always under the influence of CSPGs and yet there are no studies focusing on identifying the effect of PTN on microglia under the influence of CSPGs. Previous studies have identified the effect of CSPGs or PTN under the influence of LPS, but in stroke the infiltrating macrophages and T cell induce a pro inflammatory environment by releasing IFN γ. In this chapter, we hypothesised that the presence of PTN will mitigate the pro-

inflammatory activity of microglia either unstimulated or IFN γ stimulated microglia in the presence of CSPGs. To test this hypothesis, microglia were isolated from mixed glia cultures prepared from 2 - day old CX3CR1/GFP mice, cultured on CSPGs matrix, and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM). Proinflammatory cytokines released from different treatment conditions were measured.

Materials and Methods

Matrices preparation:

For the OPCs study, 12 mm coverslips (Fisherbrand, 12-545-81) were coated with 100 µg mL^{-1} Poly-D-Lysine (Sigma-Aldrich, P6407) for 2 hours at 37 ºC and 5% CO_2. After three washes with water, the coverslips were coated with growth inhibitory matrix – 1.0 µg mL^{-1} laminin + 5 µg mL^{-1} of CSPGs (Sigma Aldrich, CC117) for 2 hours and washed with PBS. For the microglia study, coverslips were coated with a growth permissive matrix of 100 µg mL^{-1} Poly-L-Lysine (Sigma-Aldrich, P6282) and for 2 hours or an inhibitory matrix with 5 µg mL^{-1} of CSPGs (Sigma Aldrich, CC117) at 37 ºC and 5% CO_2 (for OPCs study). After three washes with water, the coverslips were coated with inhibitory matrix-1.0, 2.5, 5 µg mL^{-1} of CSPGs for 2 hours and washed 2 times with PBS.

Primary OPC cell culture:

All animal protocols were conducted in accordance with the Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. Mice primary OPC cell cultures were isolated from 2-day-old CX3CR1-GFP mice. The cortices were dissected and digested with TrypLE (Gibco, 12605-028) for 10 minutes at 37°C. The tissues were treated with 60 µg mL^{-1} of DNaseI (Sigma, DN25-100) for 5

min and centrifuged at 500 X g for 3 min. Cells were dissociated from tissue by trituration in Dulbecco's modified Eagle's medium nutrient mixture F-12 (ham) (DMEM/F12) (gibco, 11320-033) supplemented with 10% fetal bovine serum (Gibco, 12483-020) and1% Penstrep (Gibco, 15140-122). Cells were then centrifuged and cultured in T 25 flasks coated with 100 µg mL^{-1} Poly-L-Lysine (Sigma-Aldrich, P6282) at 37 °C, 5% CO_2. 2/3 media was changed every 3 days; on day 9, the flasks were placed on a shaker at 50 rpm at 37 °C, 5% CO_2 to remove any loosely adherent cells. The media was changed, and flasks were placed on a shaker at 220 rpm for 16 hours at 37 °C, 5% CO_2 to detach OPCs. The media was collected, and the media with OPCs were then incubated in a cell culture dish for 30 minutes with gentle agitation every 15 minutes. All the floating cells were OPCs, and the adherent cells were microglia. The OPCs were isolated by centrifugation at 1200 rpm for 10 min and seeded at 3 X 10^4 cells well $^{-1}$ onto the coverslips coated with different matrices in OPC proliferation medium [DMEM/F12 (Gibco, 11320-033), B27 supplement (1:50 v/v) (Gibco, 17504-044), 20 ng mL^{-1} platelet-derived growth factor (PDGF-AA, Peprotech, 100-13A-10UG), 20 ng mL^{-1} fibroblast growth factor basic (FGF basic Peprotech, 100-18B-10UG), 5 µg mL^{-1} Insulin (I6634, Sigma-Aldrich)] for 24 hours. After incubation, the medium was changed to OPC differentiation media [DMEM/F12, (gibco, 11320-033), B27 supplement (1:50 v/v) (Gibco, 17504-044), N-acetyl-L-cysteine (NAC; Sigma-Aldrich, A9165-5G), Ciliary neurotrophic factor (CNTF; Peprotech, 450-13-20UG), 30 ng mL^{-1} thyronine (3,3′,5-Triiodo-L-thyronine sodium salt; Sigma-Aldrich, T2752) and 30 ng mL^{-1} thyroxine (L-Thyroxine sodium salt pentahydrate Sigma-Aldrich, T0397) and different concentration of recombinant mouse PTN (R&D 6580-PL-050)]. The cells were incubated for 7 days with ½ media change every day. The cells were then fixed with 5% formaldehyde for 20 minutes, washed with PBS and processed for immunocytochemistry.

Primary microglia cell culture

Primary microglia cells were isolated from postnatal day 2 CX3CR1-GFP mice. The cortices were dissected and digested with TrypLE (Gibco, 12605-028) for 10 minutes at 37°C. The tissues were treated with 60 µg mL^{-1} of DNaseI (Sigma, DN25-100) for 5 min and centrifuged at 300 X g for 5 min. Cells were dissociated from the tissue by trituration in Dulbecco's modified Eagle's medium nutrient mixture F-12 (ham) (DMEM/F12) (Gibco, 11320-033) supplemented with 10% fetal bovine serum (Gibco, 12483-020) and 1% Penstrep (PS) (Gibco, 15140-122). Cells were centrifuged at 500 X g for 3 min and cultured in 12 well cell culture plates coated with 100 µg mL^{-1} Poly-L-Lysine (PLL) (Sigma-Aldrich, P6282) at 37 °C, 5% CO_2. Cells were cultured for 19 days at 37 ºC and 5% CO_2, with media changed twice weekly. On 20th day for microglia isolation, confluent cell layers were washed with sterile PBS and treated with Trypsin-EDTA (0.25%) (Gibco, 25200072) and DMEM F12 mixture (1:3) for 20 minutes. The isolated cell layer consists of a mixed cell population, and the cells attached to the plates were pure microglia. Microglia were trypsinised with 0.25% trypsin-EDTA for 10 min, centrifuged at 1200 rpm for 10 min and seeded at 1 X 10^5 cells/well onto coverslips coated with different matrices for 24 h in DMEM F12/1%PS. After 24 h, the media was changed with media containing different concentrations of recombinant mouse PTN (R&D 6580-PL-050) for 72 h. The media was collected after 72 h and analysed for cytokine levels using a pro-inflammatory focused 10 plex discovery assay (Eve Technologies, Calgary, AB) and the cells were fixed after 72 h of treatment with 5% formaldehyde solution for 15 min., washed with PBS and processed for immunocytochemistry.

EdU proliferation assay

Microglia proliferation was measured by EdU analysis using Click-iT EdU kit (Thermo Fisher Scientific, C10638). Briefly, microglia were added onto coverslips coated with Poly-L-

Lysine as a control group and with different concentrations of CSPGs (1, 2.5 and 10 μg mL^{-1}). After 24 h of incubation, the media was changed with media containing 20 μM EdU and different concentrations of PTN with/without 100 μg mL^{-1} IFN. Cells were fixed after 72 h with 5 % formaldehyde for 15 min. and washed with PBS 3 X. Following fixation, the cells were permeabilised with 0.5 % Triton X-100. The Click-iT mix solution was added to each well and incubated for 30 min. Following incubation, the cells were washed twice with 0.1% triton X-100 in PBS. The cells were then processed for Iba1 immunostaining.

Gel zymography

Conditioned media from cell culture were centrifuged to remove cell debris and concentrated using an amicon ultra-2 centrifugal filter unit (Millipore UFC201024). MMP activity was determined by gel zymography using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with modification. The samples were run on 7.5% acrylamide gel with 0.1% (w/v) gelatin. 15 ug protein was loaded into each well, diluting 5 X sample buffer to 1 X buffer and run under non-reducing conditions at 110 V and 4 °C for 2 h. After electrophoresis, gels were washed with wash buffer (2.5 % Triton X 100, 50 mM Tris HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) for 2 X 30 min with agitation. The gels were then incubated in incubation buffer (1.0 % Triton X 100, 50 mM Tris HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) for 24 h at 37 °C. The gels were then stained with 0.5% Coomassie blue (0.5% Coomassie blue, 50% water, 10% acetic acid, 40 % methanol) for 1 h. The gels were then destained with destaining solution (40% methanol, 10% acetic acid, 50% water) until white bands were detected under a blue background. Gels were then imaged using a Licor Odyssey reader. The bands were analysed using a gel analyser plugin in Fiji [230].

Immunocytochemistry

The fixed samples were blocked with 10% normal horse serum in PBS containing 0.1% Tritton-X 100 and washed with PBS. Samples were stained overnight with Myelin basic protein antibody (MBP) (1:500, Merk Millipore MAB386), oligodendrocyte marker 4 (O4) (1:500, R&D MAB1326), platelet-derived growth factor α (PDGFRα) (1:500, R&D AF1062), Chondroitin sulfate proteoglycans (CSPGs) CS56 (1:500, sigma C8035) and Iba1 (1:500, ThermoFisher Scientific PA5-27436) (PBS pH 7.4 with 0.1% HS, overnight at 4° C). The primary antibody CS56 and O4 was detected with secondary antibody donkey anti-mouse Alexa 647 (1:500 abcam ab150107), MBP was detected with donkey anti-rat Alexa 488 (1:500 life technologies A21208), PDGFRα was detected with donkey anti-goat Alexa 546 (1:500 life technologies, A11056), Iba1 was detected with secondary antibody goat anti-rabbit Alexa 488 (1:500 Thermofisher Scientific A-11034) nucleus was stained with Hoechst 33342 (1:1000, Invitrogen, 62249). The samples were then mounted on slides with fluormount-G (Southern Biotech). The images were taken using a Leica TCS-SPE fluorescent microscope.

Statistics

Statistical analyses of data from fluorescent images were carried out using one-way ANOVA followed by Tukey's multiple comparisons test for significance between treatment groups. For SHOLL analysis, the data were analysed using two-way ANOVA and Tukey's multiple comparisons test for significance between treatment groups. n represents a single independent experiment (i.e. an independent culture preparation) with a minimum of three technical replicates. Each technical replicate represents a well in a 24-well culture plate. All image analyses were performed by experimenters blind to experimental conditions, and statistical analyses were done using Graphpad Prism version 9.1.2.

Results

PTN drives differentiation of OPCs to oligodendrocytes, overcoming the inhibitory effect of CSPGs

To determine the effect of PTN on the differentiation of OPCs in the presence of CSPGs, we coated coverslips with Laminin and CSPGs. The isolated OPCs were seeded onto the matrix and cultured in the presence or absence of different concentrations of PTN (1 nM, 10 nM, 100 nM) for 7 days. To detect the differentiation of OPCs to oligodendrocytes, the cells were fixed after 7 days of treatment and immunolabelled with different markers for oligodendrocyte lineage (OPCs, premyelinating oligodendrocytes and differentiated/mature oligodendrocytes). During the differentiation of OPCs to oligodendrocytes, protein expression gradually changes with OPCs expressing PDGFRα, premyelinating oligodendrocytes expressing O4 and matured oligodendrocytes expressing MBP (Figure 3.1 A). OPCs tend to differentiate and express O4 even in the presence of CSPGs (Figure 3.1 E) but cannot differentiate to oligodendrocytes expressing MBP (Figure 3.1 F). PTN restored the differentiation of OPCs to oligodendrocytes in a dose-dependent manner. OPCs differentiated to oligodendrocyte expressing O4 and MBP upon treatment with 1 nM PTN (Figure 3.1 G – K), 10 nM PTN (Figure 3.1 L – P) and 100 nM PTN (Figure 3.1 Q – U).

PTN treatment significantly reduced PDGFRα positive cells (ANOVA, $F_{(3, 8)} = 7.831$ $P = 0.0091$) (Figure 3.2 A), and post hoc comparisons identified that 10 nM PTN treatment significantly reduced the number of PDGFRα positive cells compared to 1 nM PTN (Tukey's $P = 0.0496$) while 100 nM PTN significantly increased PDGFRα positive cells time (Tukey's $P = 0.0138$). PTN treatment significantly reduced the number of cells positive for PDGFRα + O4 positive cells ($F_{(3, 8)} = 5.635$ $P = 0.0226$) (Figure 3.2 B), and post hoc comparisons identified that

100 nM PTN treatment significantly reduced the number of PDGFRα and O4 positive cells compared to non-treated condition (Tukey's $P = 0.0145$). The premyelinating oligodendrocyte population expressing O4 showed a significant increase with the PTN treatment ($F_{(3, 8)} = 21.59$ $P = 0.0003$) (Figure 3.2 C), and post hoc analysis showed that 10 nM PTN significantly increased O4 positive cell population compared to 1 nM PTN (Tukey's $P = 0.0135$) and 100 nM PTN (Tukey's $P = 0.0339$). MBP expressing mature oligodendrocytes showed a significant increase with PTN treatment ($F_{(3, 8)} = 13.17$ $P = 0.0018$) (Figure 3.2 D), and post hoc analysis showed that 10 nM PTN significantly increased MBP positive cell population compared to 1 nM PTN (Tukey's $P = 0.00195$). In the absence of PTN, there was no significant difference in the cell population expressing PDGFRα (46.04% of the total cell population) and cells positive for PDGFRα and O4 (43.93% of total cell population) but there was significantly fewer premyelinating O4 expressing cells (9.32% of total cell population) ($F_{(3, 8)} = 12.57$ $P = 0.0021$) (Figure 3.2 E). With 1 nM PTN treatment, a fraction of O4 expressing premyelinating oligodendrocyte increased ($F_{(3, 8)} = 18.61$ $P = 0.0006$) (Figure 3.2 F). With the treatment of 10 nM PTN, the fraction of O4 expressing premyelinating oligodendrocyte significantly increased (46.84% of the total cell population) compared to PDGFRα expressing OPCs (11.98% of the total cell population) ($F_{(3, 8)} = 28.30$ $P = 0.0001$) (Figure 3.2 G). In 100 nM PTN treatment, the fraction of PDGFRα cell population (45.54% of the total cell population) significantly increased compared to O4 expressing cell (30.55% of the total cell population) ($F_{(3, 8)} = 66.53$ $P < 0.0001$) (Figure 3.2 H). The above data suggests that PTN drives the differentiation of OPCs to oligodendrocytes, overcoming the inhibitory effect of CSPGs.

Effect of PTN on process outgrowth from OPCs and oligodendrocytes.

To determine the ability of PTN to increase complexity in the outgrowth from OPCs, premyelinating oligodendrocytes and oligodendrocytes, OPCs were isolated from mice pups, cultured on laminin + CSPGs matrix, and treated with different concentrations of PTN (1 nM PTN, 10 nM PTN, 100 nM PTN). The cells were immunostained for PDGFRα, O4 and MBP, and images were then analysed for cellular outgrowth using Sholl analysis from Fiji [271]. Intersections were counted at every 2 μm as shown in (Figure 3.3 A). The PTN treatment significantly increased the outgrowth from PDGFRα positive cells (Figure 3.3 B), O4-positive cells (Figure 3.3 C) and MBP-positive cells (Figure 3.3 D). Two-way ANOVA showed a significant interaction between distance from soma and treatment for PDGFRα positive cells ($F_{(141, 384)} = 1.757$ $P < 0.0001$), O4 positive cells ($F_{(183, 496)} = 1.9$ $P < 0.0001$), MBP positive cells ($F_{(153, 416)} = 1.290$ $p = 0.0249$). A significant effect on intersections distance from soma for PDGFRα positive cells ($F_{(47, 384)} = 59.84$ $P < 0.0001$), O4 positive cells ($F_{(61, 496)} = 19.29$ $P < 0.0001$) and MBP positive cells ($F_{(51, 416)} = 15.14$ $P < 0.0001$). A significant effect of treatment on number of intersections for PDGFRα positive cells ($F_{(3, 384)} = 79.15$ $P < 0.0001$), O4 positive cells ($F_{(3, 496)} = 163.1$ $P < 0.0001$) and MBP positive cells ($F_{(3, 416)} = 94.29$ $P < 0.0001$). Tukey's post hoc test revealed that with PTN treatment, there was a significant change in the number of intersections for PDGFRα positive, O4 and MBP-positive cells within the following mentioned distance from soma (Figure 3.3 B – C):

Table 2: Radius process outgrowth from PDGFR α, O4 and MBP positive cells showing significant difference in number of intersections with PTN treatment.

	PDGFR α	O4	MBP
PTN conc. (nM)	Distance form soma (µm)	Distance form soma (µm)	Distance form soma (µm)
1	7 – 25	13 – 43	15 – 35
10	7 – 29	13 – 55	15 – 65
100	7 - 39	13	41 – 53

Post hoc analysis also showed that for O4-positive cells, there was a significant change in the number of intersections between 1 nM PTN and 10 nM PTN treatments within 17 µm – 45 µm, between 10 nM PTN and 100 nM PTN treatments within 31 µm – 45 µm (Figure 3.3 C). To confirm these findings, we further measured the total number of intersections for each treatment condition for PDGFRα positive cells (Figure 3.3 E), O4 positive cells (Figure 3.3 F), and MBP positive cells (Figure 3.3 G). PTN treatment significantly increased the number of intersections for PDGFRα positive cells ($F_{(3, 8)} = 5.732$ $P = 0.0216$), O4 positive cells ($F_{(3, 8)} = 7.444$ $p = 0.0106$) and MBP positive cells ($F_{(3, 8)} = 5.147$ $p = 0.0284$) and post hoc analysis showed that 10 nM PTN treatment condition significantly increased number of intersections for PDGFR α ($P = 0.0253$), O4 ($P = 0.0078$) and MBP ($P = 0.0261$) compared to CSPGs control. Thus, the data indicate that 10 nM PTN concentration induces growth of OPCs and induces differentiation to oligodendrocytes.

Effect of PTN on proliferation and release of pro-inflammatory cytokines from microglia in the presence of CSPGs

The cytokines released form microglia can also influence the proliferation and differentiation of OPCs to oligodendrocytes. During an injury the microglia become activated and release cytokines. In this study, released cytokines were measured to determine the effect of PTN on microglia in the presence of CSPGs. Isolated microglia from mixed glial cell culture were first tested for their purity, and based on immunostaining with Iba1, the isolated cell population was 95% microglia (Figure 3.4 A-C). The microglia were then cultured on a CSPGs matrix (5 µg mL^{-1}) and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM PTN) for 72 h. The media was collected and processed to detect the following cytokines (TNF, IL 6, IL 1B, MCP and IL 10). The data indicate that CSPGs reduce the release of pro-inflammatory cytokines, and with PTN treatment, the expression of pro-inflammatory cytokines was further reduced. In the presence of CSPGs, PTN treatment significantly reduced the expression of TNF cytokine ($F_{(3, 8)} = 70.96$ $P < 0.0001$); and post hoc analysis further showed that the expression of TNF with 1nM PTN treatment was higher than 10 nM PTN (Tukey's $P = 0.0011$) and 100 nM PTN (Tukey's $P = 0.0461$) (Figure 3.4 D). The release of IL 6 was significantly reduced with PTN treatment in the presence of CSPGs ($F_{(3, 8)} = 87.51$ $P < 0.0001$); and post hoc analysis showed that the expression of IL 6 with 1nM PTN treatment was higher than 10 nM PTN (Tukey's $P = 0.0091$) and 100 nM PTN (Tukey's $P = 0.0481$) (Figure 3.4 E).

Similarly, PTN treatment also reduced the expression of IL 1B in the presence of CSPGs ($F_{(3, 8)} = 42.89$ $P < 0.0001$), though post hoc analysis did not show any difference in different doses of PTN (Figure 3.4 F). MCP 1 is also a pro-inflammatory cytokine; PTN treatment reduced the expression of MCP 1 in the presence of CSPGs ($F_{(3, 8)} = 73.51$ $P < 0.0001$), and post hoc analysis

also showed that 1 nM PTN treatment expressed higher MCP 1 compared to 10 nM PTN (P = 0.0030) and 100 nM PTN (P = 0.0389) (Figure 3.4 G). In the presence of CSPGs, PTN treatment reduced the expression of IL 10 ($F_{(3, 8)} = 87.51$ $P < 0.0001$), and post hoc analysis also showed that 1nM PTN treatment expressed higher MCP 1 compared to 10 nM PTN (P = 0.0091) and 100 nM PTN (P = 0.0481) (Figure 3.4 H). In the presence of CSPGs, there was a significant effect of treatment on the proliferation of microglia ($F_{(3, 8)} = 32.18$ $P < 0.0001$), and post hoc analysis showed that with 1 nM PTN there was an increased proliferation compared to 10 nM (Tukey's P = 0.0039) and 100 nM (Tukey's P = 0.0009) (Figure 3.4 I). All the above data showed that in the presence of CSPGs, PTN reduced the expression of proinflammatory cytokines and proliferation at higher concentrations (10 nM PTN, 100 nM PTN).

Effect of PTN on proliferation and release of pro-inflammatory cytokines from microglia in the presence of CSPGs after inflammatory stimulation

To determine the effect of PTN on microglia in the presence of CSPGs in inflammatory conditions, microglia were cultured on CSPGs matrix (5 μg mL^{-1}) and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM PTN) with 100 ng mL^{-1} IFN γ for 72 h. The media was collected and processed to detect cytokine levels (TNF, IL 6, IL 1B, MCP and IL 10). The data indicate that in the presence of IFN γ, CSPGs reduced the release of pro-inflammatory cytokines, and with PTN treatment, the expression of pro-inflammatory cytokines is increased. PTN treatment on IFN γ stimulated microglia in the presence of CSPGs significantly increased the expression of (A) TNF cytokine ($F_{(4, 10)} = 83.65$ $P <$ 0.0001), and post hoc analysis further showed that the expression of TNF with 1nM PTN treatment was lower than 10 nM PTN (Tukey's $p <$ 0.0001) and 100 nM PTN (Tukey's $P <$ 0.0001) (Figure 3.5 A), (B) IL 6 ($F_{(4,10)} = 95.57$ $p <$ 0.0001), and post hoc analysis showed that the expression of IL 6 with 1nM PTN treatment

was lower than 10 nM PTN (Tukey's $P < 0.0001$) and 100 nM PTN (Tukey's $P < 0.0001$) (Figure 3.5 B), (C) IL 1B ($F_{(4,10)} = 53.29$ $P < 0.0001$), and post hoc analysis showed that the expression of IL 6 with 1nM PTN treatment was lower than 10 nM PTN (Tukey's $P < 0.0001$) and 100 nM PTN (Tukey's $P < 0.0001$) (Figure 3. F), (D) MCP – 1 expression did not show any change with PTN treatment. (E) IL 10 ($F_{(4,10)} = 102.5$ $P < 0.0001$), and post hoc analysis showed that the expression of IL 10 with 1nM PTN treatment was lower than 10 nM PTN (Tukey's $P < 0.0003$) and 100 nM PTN (Tukey's $P = 0.0002$). (F) The IFN γ stimulated microglia showed a significant increase in proliferation ($F_{(4, 10)} = 7.626$ $P = 0.0044$) and post hoc analysis showed that 100 nM PTN treatment significantly increased the proliferation compared to 1nM PTN ($P = 0.0078$). These data suggest that in the presence of CSPGs, higher concentrations of PTN (10 nM PTN, 100 nM PTN) increased the release of proinflammatory cytokines from IFN γ activated microglia.

Expression of MMPs from PTN-treated microglia in the presence of CSPGs

MMPs are proteolytic enzymes released from cells that modify the extracellular matrix environment. Activated microglia and macrophage expresses MMPs during infection [272, 273, 265]. After 72 h of treatment, the media from different treatment concentrations were collected, and the cells were then fixed with 5% formalin and immunostained for CSPGs. The microglia cultured on PLL control matrix (Figure 3.6 A – C), microglia were cultured on the CSPGs matrix, CSPGs staining indicate that microglia release MMPs that can degrade CSPGs leaving black trails around the microglia (Figure 3.6 D – F). With PTN treatment, the unstained area (black area) surrounding microglia increased at 1 nM PTN treatment (Figure 3.6 G – I), followed by 10 nM PTN (Figure 3.6 J – L) and was highest in 100 nM PTN (Figure 3.6 M – O). To further confirm the involvement of the released MMPs from microglia cleaving off the CSPGs in the matrix, the conditioned media was concentrated using ampicon and then subjected to SDS PAGE under non-reducing conditions

in gels with 0.1% (w/v) gelatin. The gels were then incubated to determine the proteolytic activity of MMP 9 and MMP 2. The gels were stained with Coomassie Brilliant Blue, and white bands corresponding to MMP 9 and MMP 2 molecular weight were observed in a dark background (Figure 3.7 A, D). There was a significant effect of treatment on the amount of pro-MMP 9 released from microglia ($F_{(3, 8)} = 250.7$ $P < 0.0001$), and post hoc tests showed that higher concentration of PTN (10 nM PTN, 100 nM PTN) significantly reduced the release of pro-MMP 9 compared to 1 nM PTN (Tukey's $P < 0.0001$) (Figure 3.7 B). There was a significant effect of treatment on the expression of active MMP 9 ($F_{(3, 8)} = 38.54$ $P < 0.0001$), and post hoc analysis suggests that there is a dose-dependent increase in the expression of MMP 9 with 100 nM PTN showing the significant expression (Tukey's $P = 0.0070$). There was no significant change in the expression of pro-MMP 2 with PTN treatment (Figure 3.7 E), but there was a significant difference in the expression of active MMP 2 with PTN treatment ($F_{(3, 8)} = 6.154$ $P = 0.0179$), and post hoc analysis showed that 10 nM PTN induced a significant increase in the release of active MMP 2 (Tukey's $P = 0.0332$) (Figure 3.7 F). IFN γ is known to inhibit the release of MMP 9 [274]. We wanted to determine the effect of PTN on the release of MMP from IFN γ stimulated microglia. There was a significant effect of treatment on the release of pro MMP 9 ($F_{(4, 10)} = 58.32$ $P < 0.0001$), and an post hoc analysis did not show any significant difference in the release of pro MMP 9 from microglia (Figure 3.8 A – B). Moreover, the active MMP 9 levels did not differ from the PTN treatment (Figure 3.8 C). Also, the release of pro MMP 2 and active MMP 2 did not show any difference in the release of pro MMP 2 and active MMP 2 (Figure 3.8 D – F). Thus, the data suggests that in the absence of inflammatory stimulus, PTN increases the release of MMP 9 and MMP 2 in the presence of CSPGs. However, during inflammatory stimulation, PTN does not alter the release of MMPs from microglia in the presence of CSPGs.

Discussion

PTN overcomes CSPGs' inhibitory effects on differentiation of OPC

CSPGs are one of the major components of the extracellular matrix in CNS [50]. CSPGs are known to inhibit the migration of OPC, differentiation of OPCs to mature oligodendrocytes and myelination [158, 275, 261]. Our data supports previous data suggesting that in the presence of CSPGs, OPCs differentiate until they reach premyelinating oligodendrocytes expressing O4 protein [255] but cannot further differentiate to mature oligodendrocytes. Inhibition of differentiation to MBP expressing oligodendrocytes is apparent with a lack of process outgrowth. As there are undifferentiated OPCs present surrounding the lesion area that could be harnessed to improve remyelination, there is a need to identify compounds that can overcome the inhibitory nature of CSPGs and induce differentiation into mature oligodendrocytes. In this study, we tried to identify the effect of PTN on the differentiation of OPCs to oligodendrocytes in the presence of inhibitory CSPGs. Previously, it has been shown that PTN can differentiate the OPCs to mature oligodendrocytes [276]. In this study, we found that OPCs cultured on CSPGs matrix upon treatment with different concentrations of PTN even with the lowest concentration of PTN (1 nM), restored the OPCs process outgrowth. OPCs, when treated with higher concentrations of 10 nM PTN and 100 nM PTN, showed an increased expression of O4 and MBP with complex cellular process outgrowth morphology resembling differentiated oligodendrocytes, suggesting that at higher concentrations of PTN, OPCs differentiate to oligodendrocytes even in the presence of CSPGs. The percentage of PDGFR α cells in 10 nM PTN treated conditions were 9.32%, which increased to 45.54% in 100 nM PTN treated condition indicating that 10 nM PTN concentration favours differentiation of OPCs by directly binding to PTPRZ1 [268] and at 100 nM PTN concentration favours proliferation and differentiation of OPCs by inactivating PTPRZ1 signalling [277]. Another

possibility for having higher number of PDGFR positive cells at 100 nM PTN concentration could be due to PTN blocking the binding of CSPGs to their receptors [222]. Thus, by blocking the signalling of CSPGs, more OPCs can remain attached to the matrix as CSPGs signalling disrupts the actin filament polymerisation and can inhibit the binding of cells to the matrix. More experiments are required to verify these findings. Thus, this data suggests that higher concentration (100 nM PTN) can promote survival and differentiation of OPCs in the presence of CSPGs.

PTN induces complex cellular outgrowth process of OPCs and oligodendrocytes.

Another key finding of this study deciphering the effect of PTN on OPCs in the presence of CSPGs is that PTN not only increased the number of differentiated cells but also induced process outgrowth. For PDGFRα positive cells with PTN treatment, the number of intersections per "Sholl circle" increased within 7 µm to 50 µm radius from the soma. In contrast, for O4 positive cells, the number of intersections increased within 13 µm to 113 µm radius from soma, suggesting enhanced cellular process outgrowth. For MBP-positive cells, the PTN treatment increased the number of intersections within 13 µm to 65 µm radius from soma. In the absence of PTN, the OPCs tend to have bipolar morphology, whereas with PTN treatment the morphology of OPCs becomes complex with extensive process outgrowth. In addition to the alterations in branching patterns, there was an increase in the number of intersections for OPCs, PDGFRα and MBP positive cells with PTN treatment higher than 1 nM PTN (Figure 3.2 D and Figure 3.3 G). Thus, these findings suggest that a 100 nM PTN concentration might be optimal to enhance differentiation of OPCs, making PTN a potential candidate to restore remyelination process in CSPGs-rich lesion areas.

PTN reduces the release of pro-inflammatory cytokines and MMP in the presence of CSPGs

Inflammation can influence the proliferation and differentiation of OPCs. IFN γ released form activated microglia induces apoptosis, delays remyelination, inhibits OPC differentiation and remyelination [278,279]. [280]Thus, modulating inflammatory response from microglia can alter the differentiation process affecting remyelination. Previous studies have shown that PTN and CSPGs can affect the inflammatory activity of microglia. PTN potentiates more pro-inflammatory phenotype in response to LPS treatment [197], while in the presence of CSPGs, microglia reduces the expression of cytotoxic TNF and NO after LPS treatment [265]. This study examined the combined effect of PTN on microglia activity in the presence of CSPGs. CSPGs reduced the expression of pro-inflammatory cytokines (TNF, IL 6, IL 1β and MCP 1). Microglia in the presence of CSPGs, when treated with PTN, further reduced their expression of pro-inflammatory cytokines (TNF, IL 6, IL 1β and MCP 1), especially at higher concentrations of PTN (10 nM and 100 nM). This indicates that PTN can be a potential agent which can reduce the release of pro-inflammatory cytokines during the chronic phase of injury. Notably, we also found that the expression of IL 10 was also downregulated with PTN treatment on microglia in the presence of CSPGs. IL 10 is a potent anti inflammatory cytokine, IL 10 attenuates neuro inflammation by reducing expression of TNF, IL 1β and IL 6 [280.] *In vitro* stimulation with LPS on microglia from IL 10 knock out mice induced higher expression of inflammatory cytokines (TNF and IL 6) [281]. Interestingly, the data generated from this study indicates that in the presence of CSPGs, PTN reduces the release of pro inflammatory cytokines along with reduced release of anti inflammatory cytokine, suggesting that microglia can posses M1 and M2 phenotypes further indicating that M1/M2 classification does not hold true.

Along with reducing the expression of pro-inflammatory cytokines, PTN treatment also induced the release of MMP 9 and MMP 2, which can potentially cleave off the CSPGs in the glial scar region or the lesion site. During the chronic phase of stroke recovery, CSPGs are expressed and can inhibit the neuronal growth and differentiation of OPCs to oligodendrocytes [50, 255, 261]. All the above-generated data suggests that PTN can be a potential therapeutic molecule during the chronic stage of injury, as in CSPGs that can reduce the inflammatory environment and create an environment that can help neurons to regenerate and OPCs to differentiate by digesting CSPGs in the glial scar.

PTN enhances release of inflammatory factors from activated microglia in the presence of CSPGs

During the acute phase of injury, inflammation is mediated by microglia and macrophages [265, 64, 65]. Inflammatory signaling is essential during the acute phase of stroke to recruit microglia and macrophages to the damaged area and restrict the spread of infarction to other parts of the brain by forming the glial scar [282]. Here, we modeled early inflammatory conditions in an injury by stimulating microglia with IFN γ [283,284]. Previous literature has shown that CSPGs reduce the release of TNF and NO after LPS stimulus [285], and our data further confirms that CSPGs also reduce the release of pro inflammatory cytokines from microglia under IFN γ stimulation. IFN γ stimulated microglia, upon treatment with higher concentrations of PTN (10 nM and 100 nM), promoted the release of pro-inflammatory cytokines PTN at (TNF, IL 6 and IL 1β) and IL 10 but did not alter levels of MCP -1. This data suggests that PTN can restore activated microglia's inflammatory behaviour after it has been reduced due to the presence of CSPGs. This data further suggests that PTN can be a useful treatment during the acute phase of stroke, restoring the inflammatory activity of microglia to clear debris restrict the spread of infarction. Microglia-

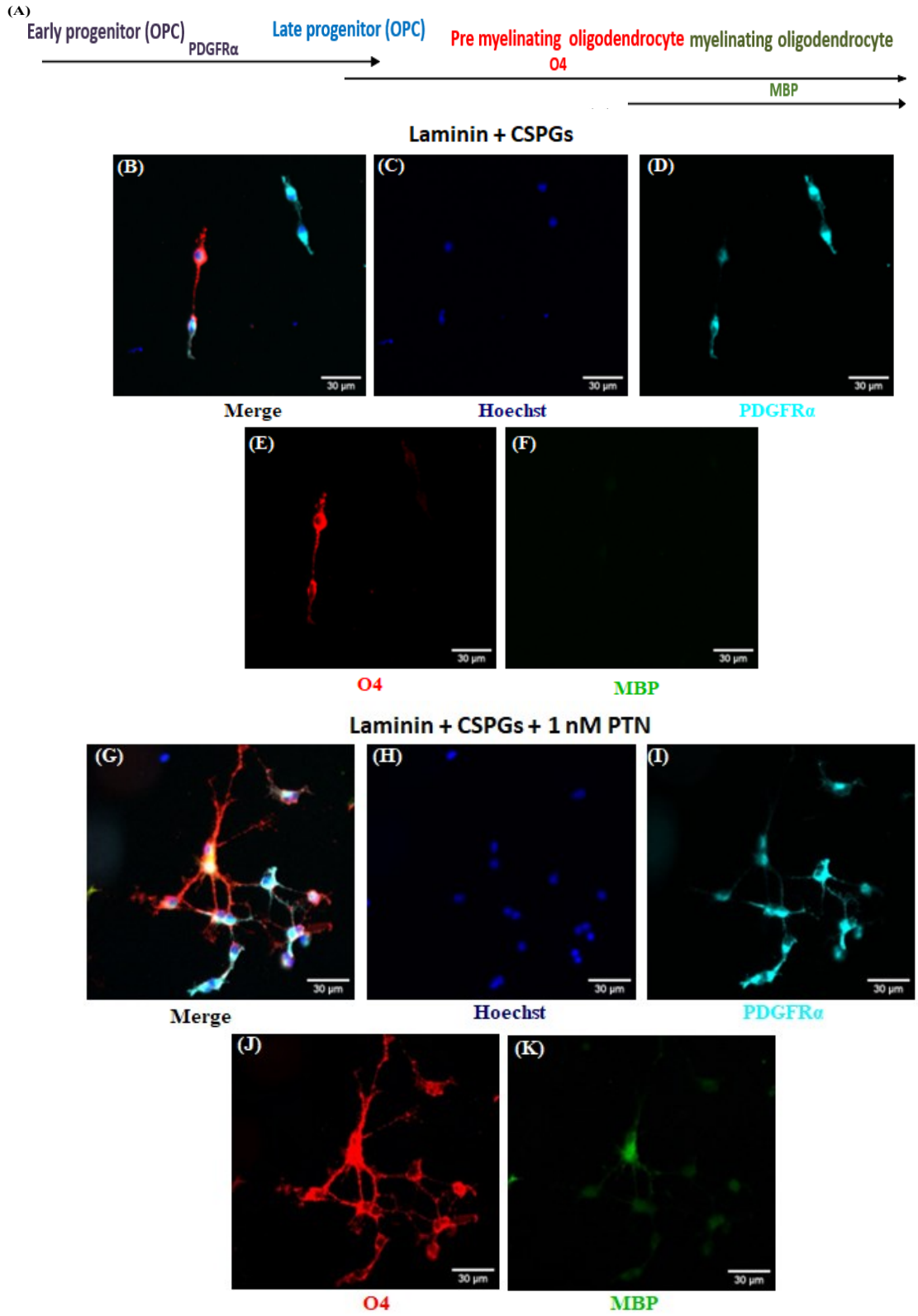
mediated release of MMP 9 degrades NG2 (one of the CSPGs) and restores oligodendrocyte differentiation from inhibitory NG2 [260]. As mentioned in the previous data, PTN treatment in the presence of CSPGs can induce the release of MMP 9 and MMP 2, but after IFN γ treatment, PTN did not increase the release of MMP 9 and MMP 2. This lack of release of MMP 9 and MMP 2 could be beneficial during the acute stage of stroke, as CSPGs play an important role in forming the glial scar, which acts as a physical barrier for the expansion of infarct. Thus, PTN can help during early stage of injury by increasing the release pro inflammatory cytokines and modulating the activity of microglia and astrocytes to form glial scar. Moreover, PTN can help the recovery process during late phase of injury by reducing the release of pro inflammatory cytokines and inducing the release of MMP 9 and MMP 2 that can cleave CSPGs creating an environment favourable for neuronal regeneration and OPC differentiation to oligodendrocytes, myelinating regenerated neurons.

Conclusion:

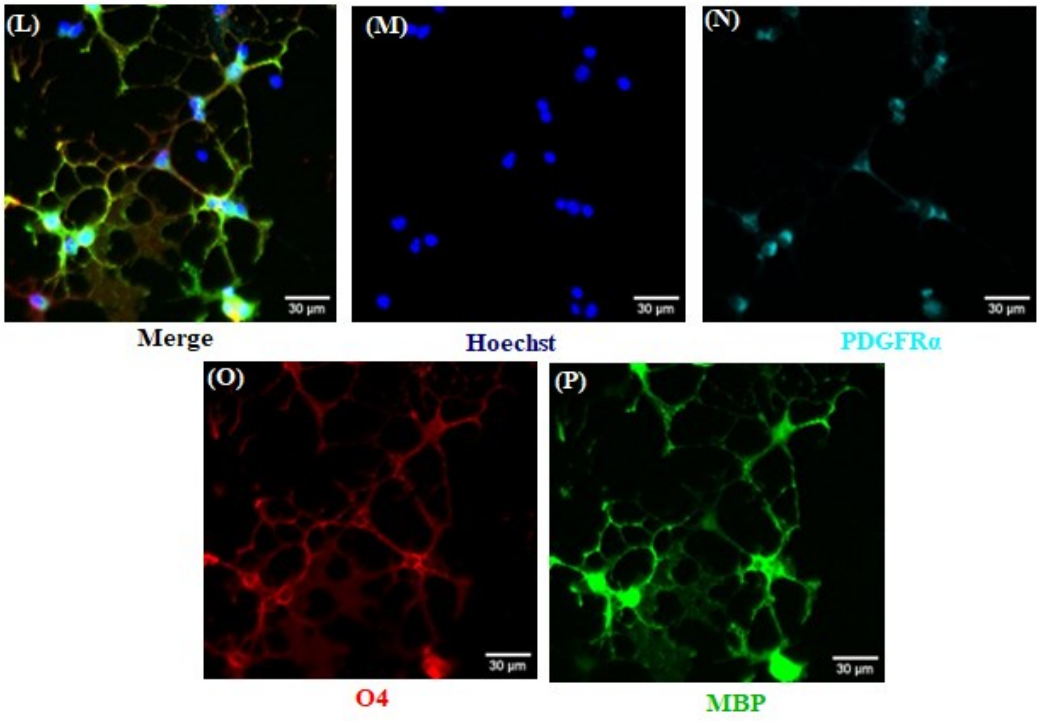
Chapter 3 investigated the effect of PTN on microglia in the presence of CSPGs during non-inflammatory and inflammatory conditions. Our study examined the dose-dependent effect of PTN and CSPGs on the differentiation of OPCs to mature oligodendrocytes. Results suggest that a higher concentration of PTN favours the adherence of OPCs to the CSPGs matrix and potentiates the differentiation of OPCs to oligodendrocytes. In the presence of CSPGs, we found that PTN has opposite effects on microglia with or without IFN γ stimulation. In IFN γ stimulated microglia, PTN enhances the inflammatory phenotype of microglia, and in the absence of IFN γ stimulation, PTN reduces the inflammatory response. In summary the data suggest the possibility that in stroke patients, during the crucial acute injury phase characterized by pro inflammatory environment, CSPGs suppress the release of pro-inflammatory cytokines. However, PTN treatment has the

ability to reverse this suppression and restore the release of these pro-inflammatory cytokines. During the chronic phase, the PTN treatment can induce the release of MMP 9 and MMP 2, which can cleave CSPGs in the glial scar, allowing the neurons to regenerate and OPCs to differentiate, reducing the release of pro-inflammatory cytokines from microglia. Thus, data generated from this chapter suggest that PTN can be a potential agent that can help the recovery process during different early (acute) and late (chronic) stage of stroke (Figure 3.9).

Figures


(A)
Early progenitor (OPC)
PDGFRα
Late progenitor (OPC)
Pre myelinating oligodendrocyte
O4
myelinating oligodendrocyte
MBP
Laminin + CSPGs
(B)
(C)
(D)
Merge
Hoechst
PDGFRα
(E)
(F)
O4
MBP
Laminin + CSPGs + 1 nM PTN
(G)
(H)
(I)
Merge
Hoechst
PDGFRα
(J)
(K)
O4
MBP
30 µm

Laminin + CSPGs + 10 nM PTN



Laminin + CSPGs + 100 nM PTN

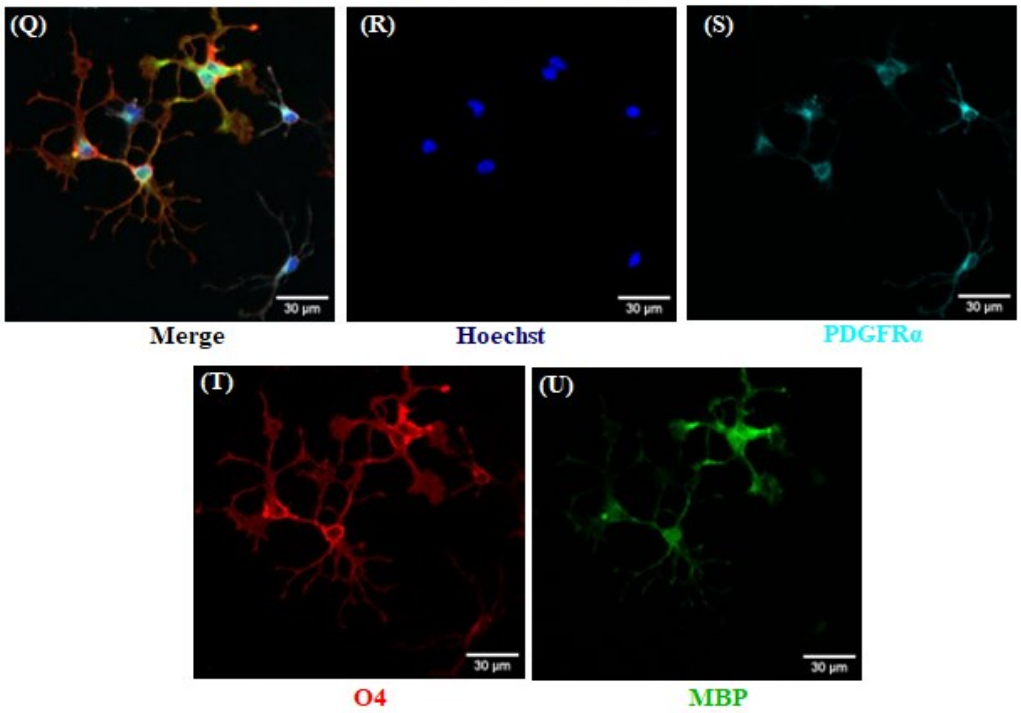
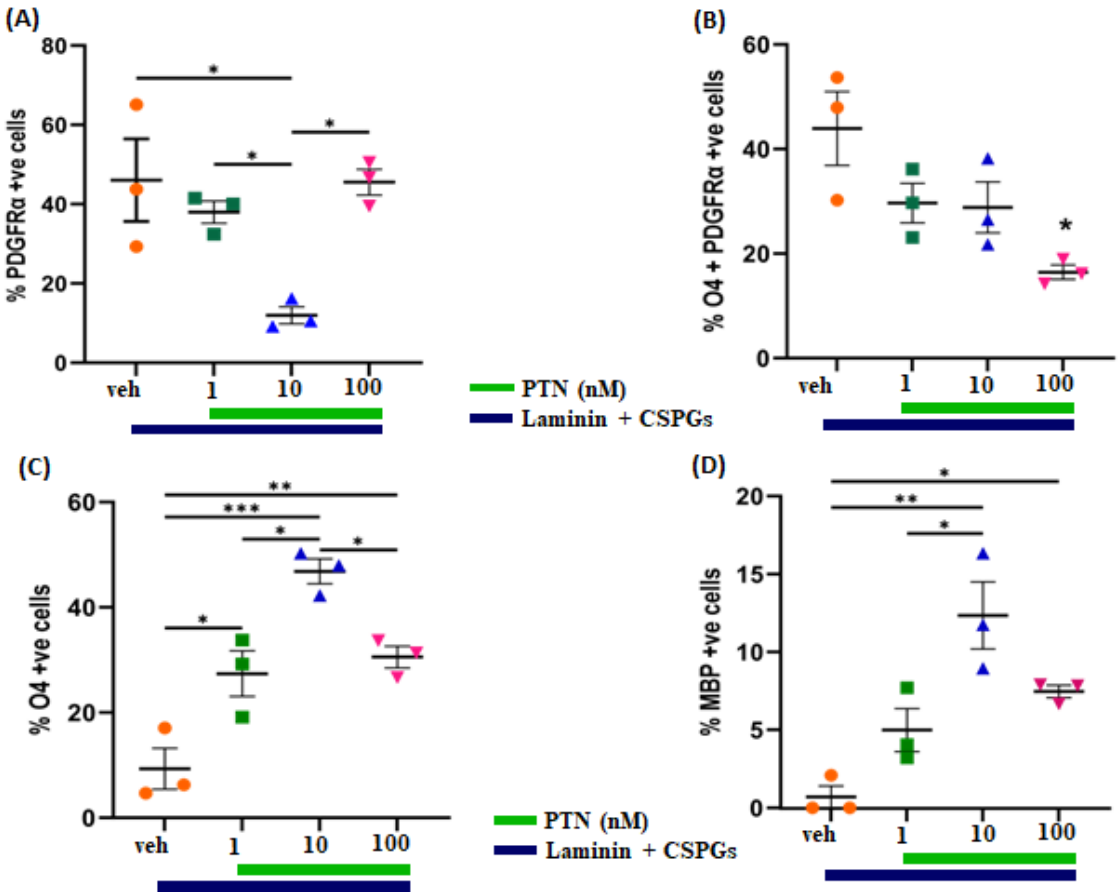


Figure 3.1: PTN induces expression of various oligodendrocyte lineage differentiation marker.

Isolated OPCs were seeded on Laminin and Laminin + CSPGs and cultured for 7 days in differentiation medium with different concentrations of PTN. Fixed cells were stained with anti-PDGFRα, anti-O4 and anti-MBP in conjunction with nuclear stain with Hoechst. (A) Markers of OPCs and oligodendrocytes during differentiation process, Epifluorescent images of differentiating OPC cultures were acquired at 20 X, OPCs were identified with marker PDGFRα (cyan), premyelinating oligodendrocytes with O4 (red), differentiating oligodendrocytes with MBP (green) and. (B – F) OPCs cultured on Laminin + CSPGs matrix, (G – K) Laminin + CSPGs matrix and treated with 1 nM PTN, (L – P) Laminin + CSPGs matrix and treated with 10 nM PTN, (Q – U) Laminin + CSPGs matrix and treated with 100 nM PTN. The scale bar represents 30 μm.


(A)
% PDGFRα +ve cells
0
20
40
60
80
veh
1
10
100
(B)
% O4 + PDGFRα +ve cells
0
20
40
60
veh
1
10
100
PTN (nM)
Laminin + CSPGs
(C)
% O4 +ve cells
0
20
40
60
veh
1
10
100
(D)
% MBP +ve cells
0
5
10
15
20
veh
1
10
100
PTN (nM)
Laminin + CSPGs

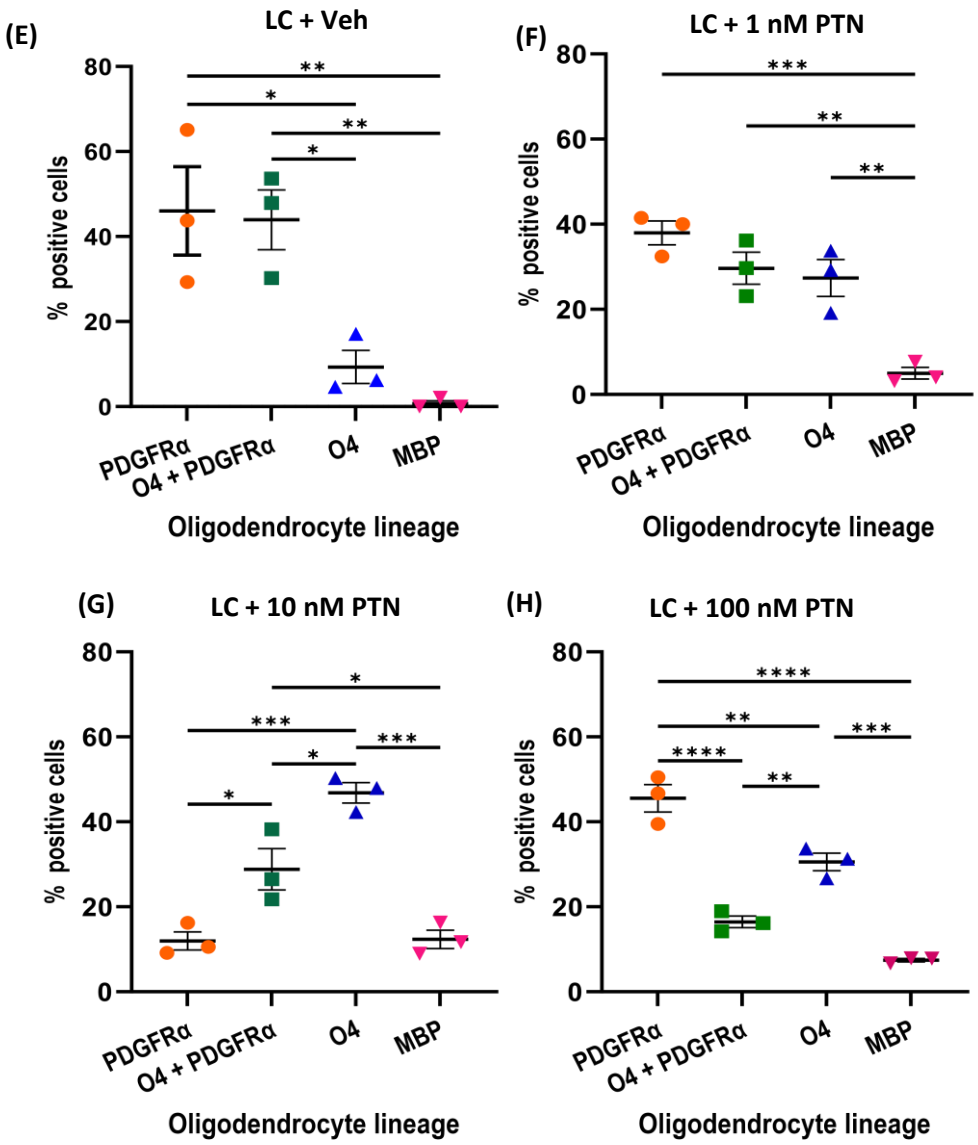
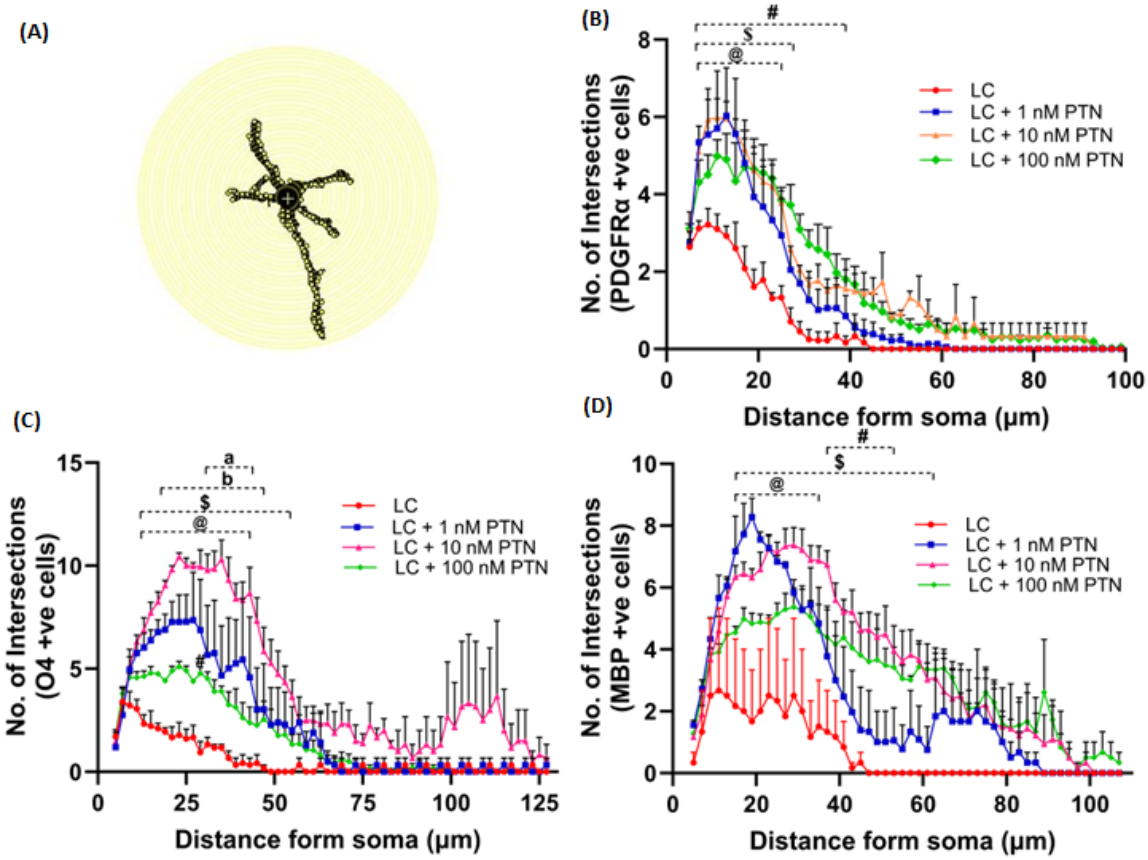


Figure 3.2: Effect of PTN on differentiation of OPCs to oligodendrocytes

OPCs cultured on Laminin + CSPGs (LC) matrix and treated with different concentrations of PTN for 7 days, the cells were stained for PDGFR α, O4 and MBP, cells positive for PDGFR α, O4 + PDGFR α, O4 and MBP were counted and plotted as percentage of total cell population. (A) PTN induces the differentiation of OPCs by reducing the number of PDGFR α cells (ANOVA, F (3, 8) = 7.831 p = 0.0091, favouring generation of (B) O4 + PDGFR α expressing cells (ANOVA, $F_{(3, 8)}$ = 5.635 p = 0.0226), (C) O4 expressing cells (ANOVA, $F_{(3, 8)}$ = 21.59 p = 0.0003), (D) MBP

expressing cells (ANOVA, $F_{(3, 8)} = 13.17$ $p = 0.0018$). Oligodendrocyte lineage markers positive cells expressing PDGFR α, O4 + PDGFR α, O4 and MBP in (E) without PTN treatment (ANOVA, $F_{(3, 8)} = 12.57$ $p = 0.0021$), (F) 1 nM PTN treatment (ANOVA, $F_{(3, 8)} = 18.61$ $p = 0.0006$) (G) 10 nM PTN treatment (ANOVA, $F_{(3, 8)} = 28.30$ $p = 0.0001$) and (H) 100 nM PTN treatment (ANOVA, $F_{(3, 8)} = 66.53$ $p <$ 0.0001). (A – H) Error bars represent the standard error of the mean (SEM). After ANOVA, Tukey's post hoc test was performed on data sets. Symbols *, ** and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of three technical replicates.



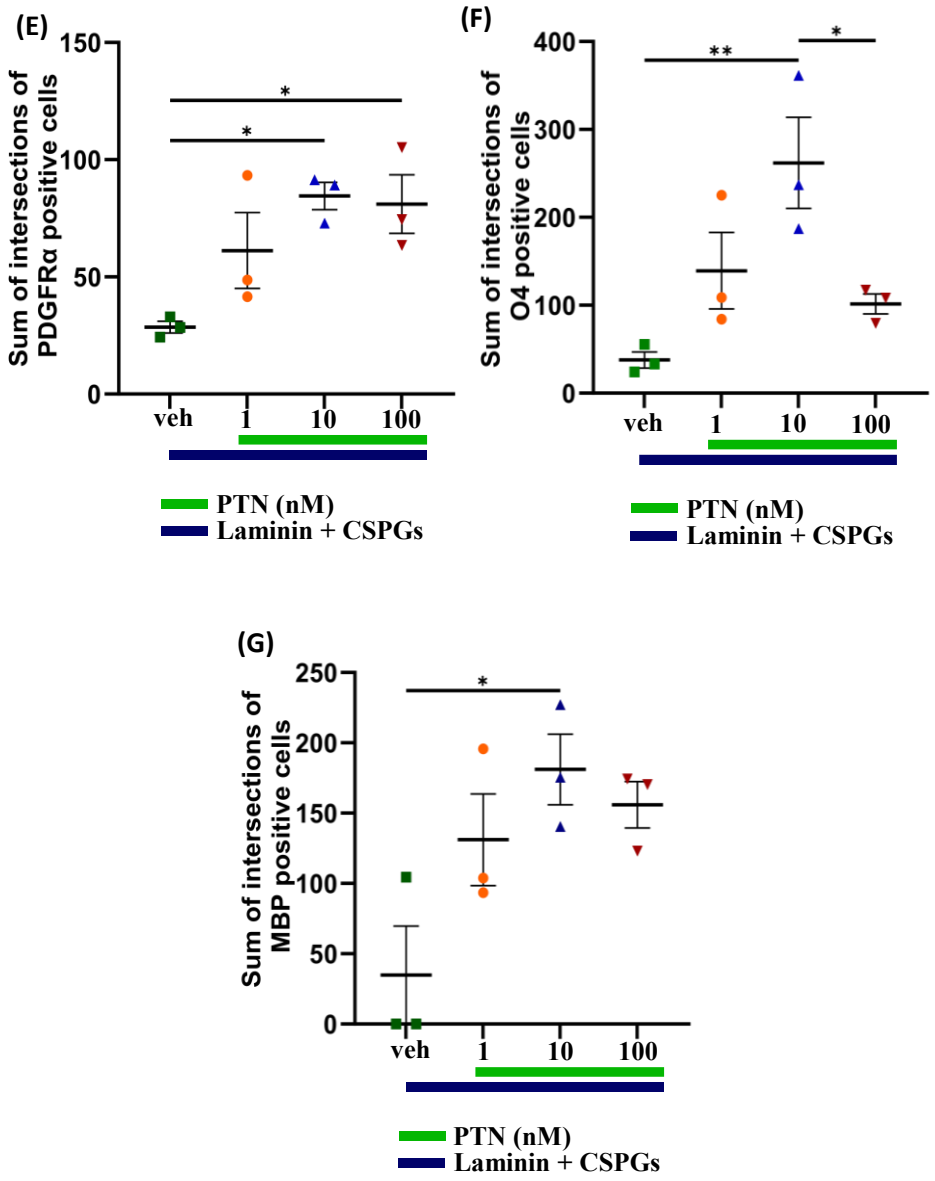


Figure 3.3: PTN promotes cellular process outgrowth from oligodendrocytes and OPCs.

(A) The schematic representation of 8 - bit image of a cell with concentric circles around cell bodies using SHOLL analysis. The yellow dots represent the intersections at that radius. (B) PTN treatment significantly increased number of intersections for PDGFR α positive cells. Two-away ANOVA identified significant interaction between number of intersections and treatment for PDGFR α positive cells ($F_{(141, 384)} = 1.757$ $p < 0.0001$), a significant main effect on number of

intersections ($F_{(47, 384)} = 59.84$ $p < 0.0001$) from soma and treatment ($F_{(3, 384)} = 79.15$ $p < 0.0001$) for PDGFR α positive cells. Tukey's post-hoc test revealed a significant difference between different concentrations of PTN (C) PTN treatment significantly increased number of intersections for O4 positive cells. Two-away ANOVA identified significant interaction between number of intersections and treatment for O4 positive cells ($F_{(183, 496)} = 1.9$ $p < 0.0001$), a significant main effect on number of intersections ($F_{(61, 496)} = 19.29$ $p < 0.0001$) from soma and treatment ($F_{(3, 496)} = 163.1$ $p < 0.0001$) for O4 positive cells. Tukey's post-hoc test revealed a significant difference between the different concentration of PTN (D). PTN treatment significantly increased number of intersections for MBP-positive cells. Two-away ANOVA identified significant interaction between number of intersections and treatment for MBP positive cells ($F_{(153, 416)} = 1.290$ $p = 0.0249$), a significant main effect on number of intersections ($F_{(51, 416)} = 15.14$ $p < 0.0001$) from soma and treatment ($F_{(3, 416)} = 94.29$ $p < 0.0001$) for MBP positive cells. Tukey's post-hoc test revealed a significant difference between different concentrations of PTN. PTN treatment significantly increased number of intersections for (E) PDGFR α positive cells ($F_{(3, 8)} = 5.732$ $p = 0.0216$) (F) O4 positive cells ($F_{(3, 8)} = 7.444$ $p = 0.0106$) and (G) MBP positive cells ($F_{(3, 8)} = 5.147$ $p = 0.0284$). (B – D) symbols represents @ - compared to LC + 1 nM PTN vs LC control, $ represents - compared to LC + 10 nM PTN vs LC control and # - compared to LC + 100 nM PTN vs LC control. (B – G) Error bars represent standard error of mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. also a, b, @, $, # represents represent $p < 0.05$. All data are based on 3 independent experiments with a minimum of three technical replicates.

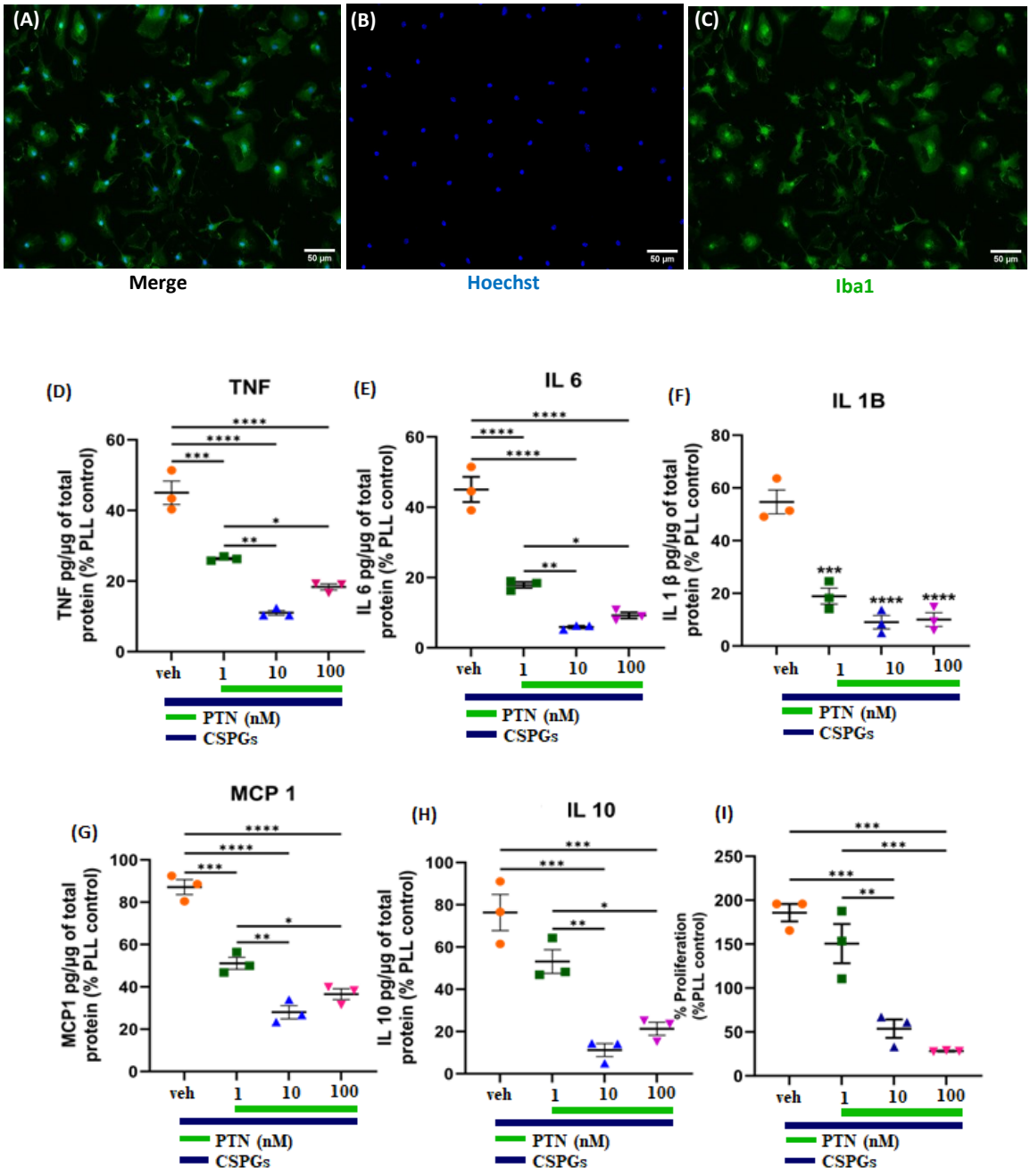


Figure 3.4: PTN treatment reduced the release of pro-inflammatory cytokines and proliferation of microglia.

(A - C) The microglia were stained with Hoechst and Iba 1. PTN significantly reduced the expression of (D) TNF ($F_{(3, 8)} = 70.96$ $P < 0.0001$), (E) IL 6 ($F_{(3, 8)} = 87.51$ $P < 0.0001$), (F) IL 1 β ($F_{(3, 8)} = 42.89$ $P < 0.0001$), (G) MCP 1 (Monocyte Chemoattractant Protein 1) $F_{(3, 8)} = 73.51$ $p < 0.0001$), (H) IL 10 ($F_{(3, 8)} = 87.51$ $p < 0.0001$), (I) ($F_{(3, 8)} = 32.18$ $p < 0.0001$). (D – I) Error bars represent the standard error of the mean (SEM), and symbols *, ** and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

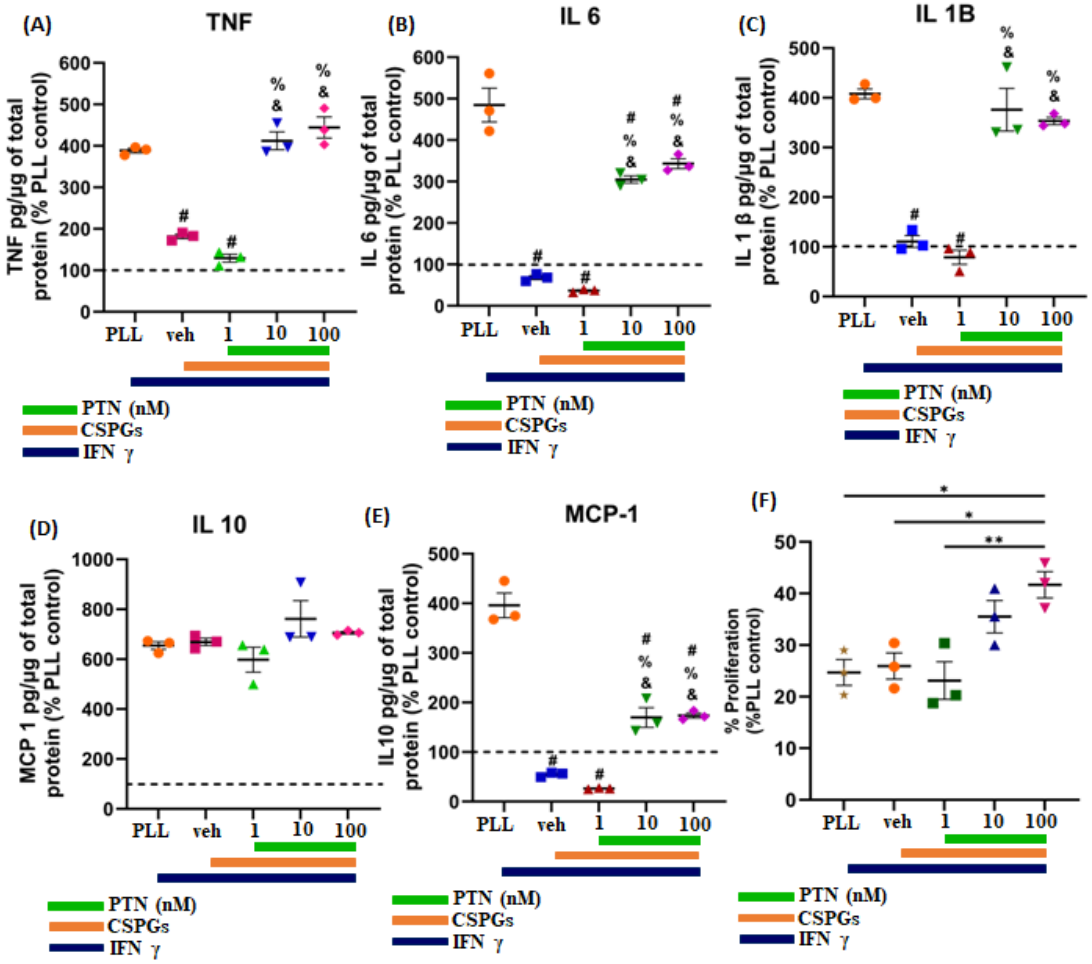


Figure 3.5: PTN treatment promoted the release of pro-inflammatory cytokines and proliferation of IFN γ stimulated microglia.

Microglia were treated with IFN γ and different concentrations of PTN for 72 h. PTN treatment increased the release of (A) TNF ($F_{(4, 10)} = 83.65$ $p < 0.0001$), (B) IL 6 ($F_{(4, 10)} = 95.57$ $p < 0.0001$), (C) IL 1 β ($F_{(4, 10)} = 53.29$ $p < 0.0001$), (D) MCP 1 ($F_{(4, 10)} = 2.244$ $p = 0.1367$), (E) IL 10 ($F_{(4, 10)} = 102.1$ $p < 0.0001$), (I) IL 10 ($F_{(4, 10)} = 29.00$ $p < 0.0001$). (F) proliferation of microglia ($F_{(4, 10)} = 7.626$ $p = 0.0044$). (A – F) Error bars represent standard error of the mean (SEM), symbols # - compared to PLL + IFN γ, & - compared to CSPGs + 1 nM PTN control and % - compared to

CSPGs control, #, &, % represents $p < 0.05$, symbols *, ** represent $p < 0.05$ and 0.01 respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

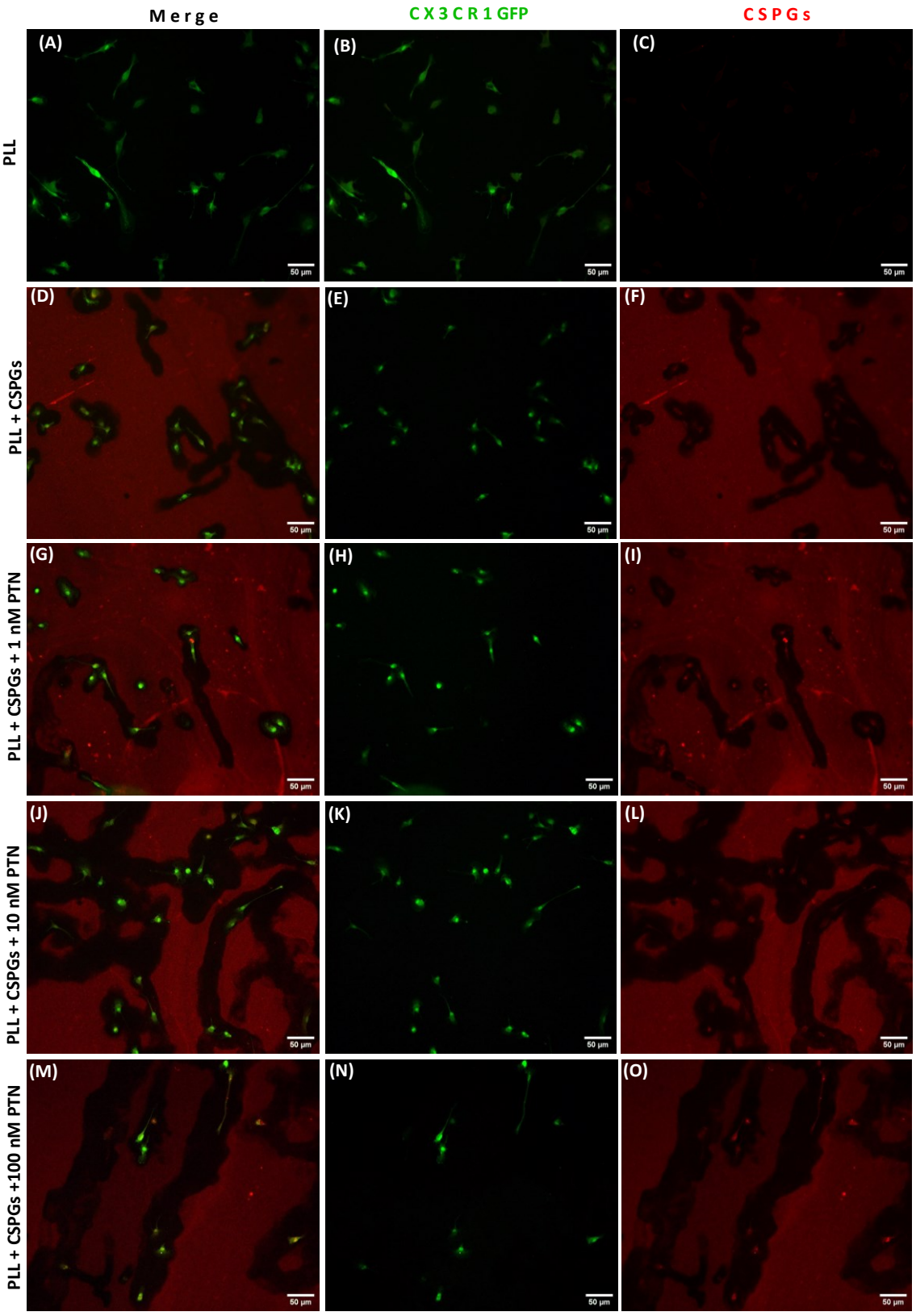
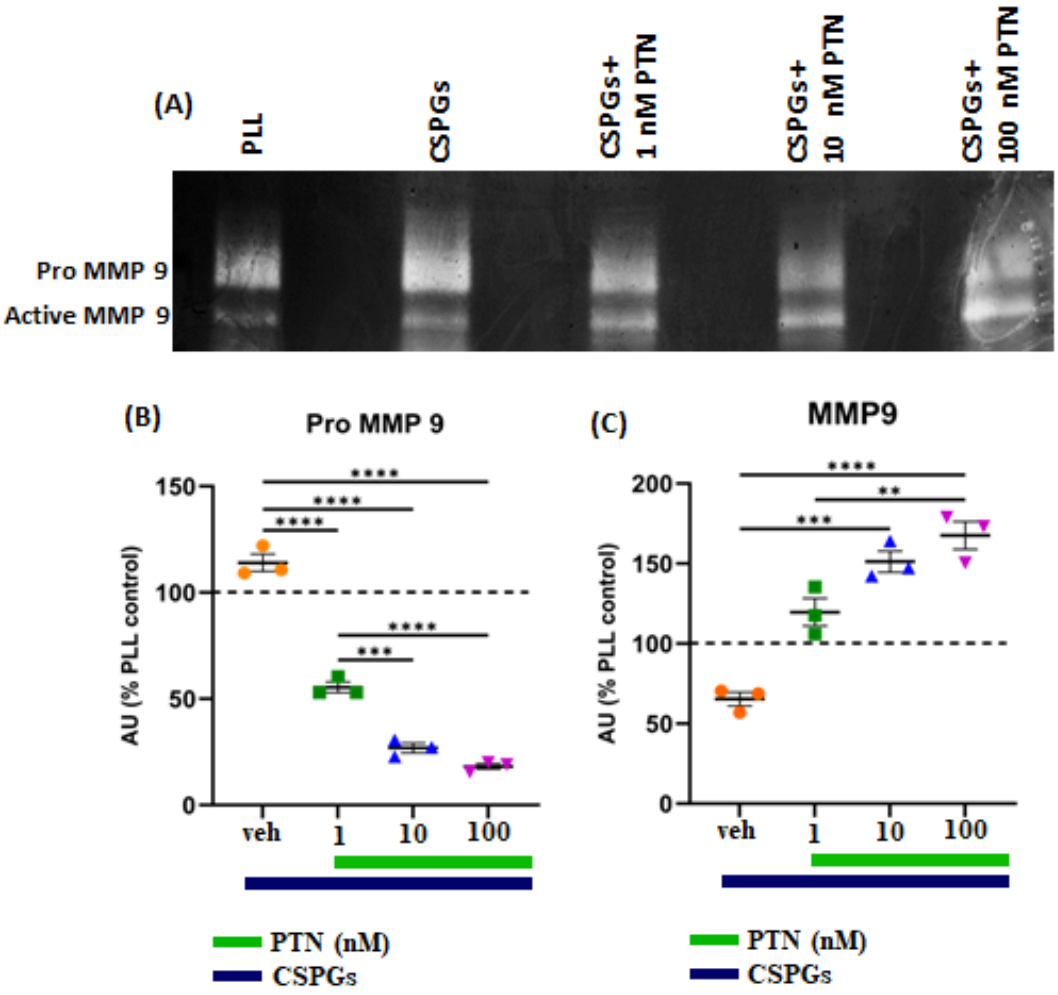

Merge
CX3CR1 GFP
CSPGs
PLL
PLL + CSPGs
PLL + CSPGs + 1 nM PTN
PLL + CSPGs + 10 nM PTN
PLL + CSPGs +100 nM PTN
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)
(L)
(M)
(N)
(O)
50 µm

Figure 3.6: PTN treatment promoted the cleavage of CSPGs by microglia.

Microglia from CX3CR1 GFP transgenic mice were cultured on CSPGs matrix, incubated with different concentrations of PTN for 72 h, stained CSPGs with CS56 antibody, and detected with Alexa 647 (red). The black area surrounding microglia are indicative of cleaved CSPGs. (A – C) Microglia cultured on poly Lysine (PLL) matrix. (D – F) Microglia cultured on CSPGs matrix. (G – I) Microglia were cultured on the CSPGs matrix and treated with 1 nM PTN. (J – L) Microglia were cultured on CSPGs matrix and treated with 10 nM PTN. (M – O) Microglia were cultured on CSPGs matrix and treated with 100 nM PTN.


(A)
PLL
CSPGs
CSPGs+ 1 nM PTN
CSPGs+ 10 nM PTN
CSPGs+ 100 nM PTN
Pro MMP 9
Active MMP 9
(B)
Pro MMP 9
AU (% PLL control)
150
100
50
0
****
****
****
****
***
veh
1
10
100
PTN (nM)
CSPGs
(C)
MMP9
AU (% PLL control)
200
150
100
50
0
****
**
***
veh
1
10
100
PTN (nM)
CSPGs

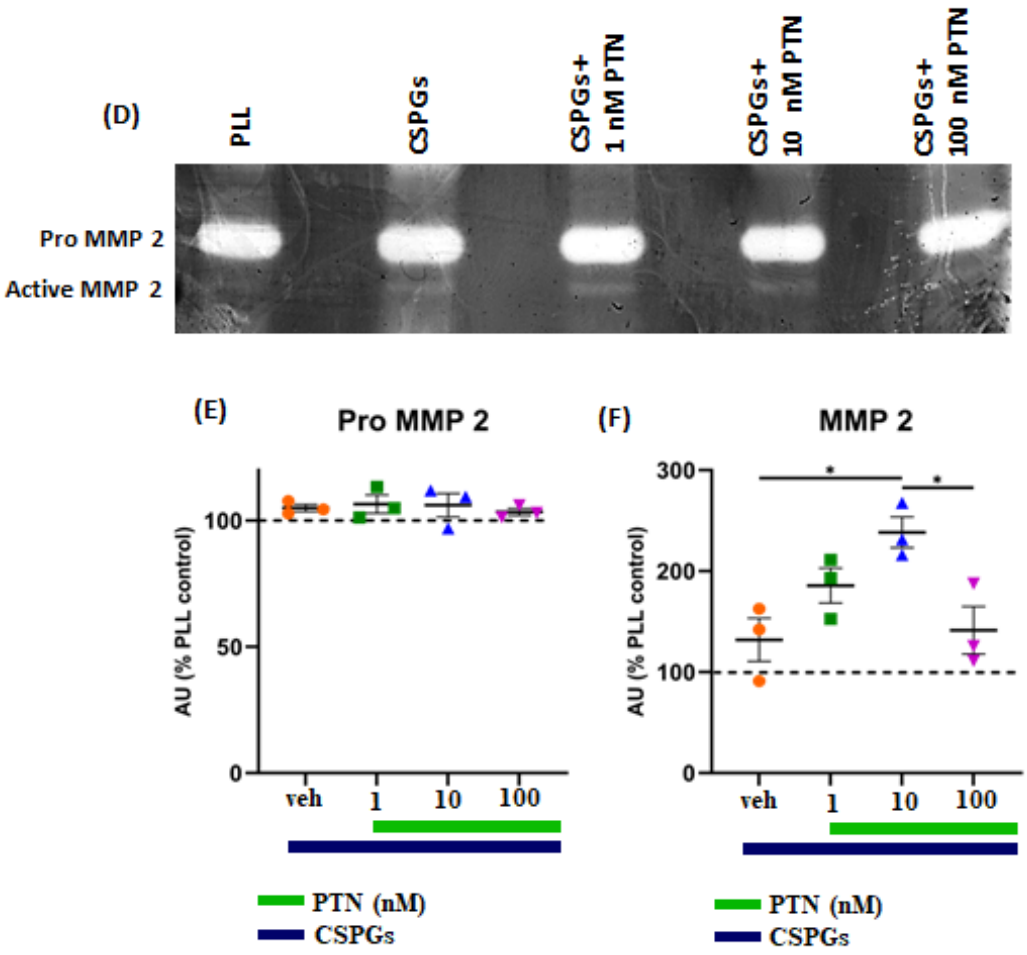
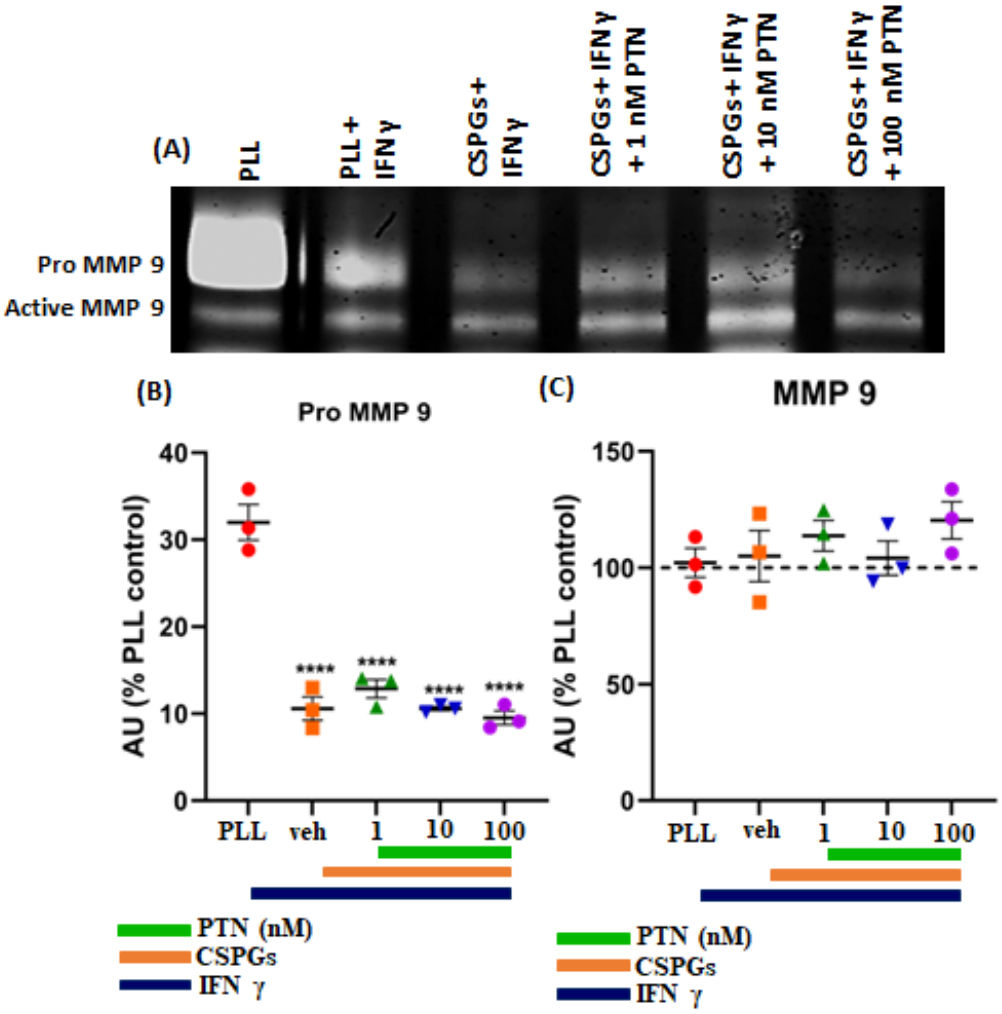


Figure 3.7: PTN treatment induced the abundance of MMP 9 and MMP 2 from microglia

MMP 9 and MMP 2 activity was detected using gel zymography from conditioned media from microglia treated with or without PTN. The intensities of the white band for (A) pro-MMP 9, active MMP 9 and (D) pro-MMP 2, active MMP 2 were calculated using image J software. The intensities of PLL control were considered 100% for pro MMP 9, active MMP 9, Pro MMP 2 and active MMP 2. (B) PTN significantly reduces the release of pro-MMP 9 from microglia ($F_{(3, 8)} = 250.7$ $p < 0.0001$) (C) PTN significantly increased active MMP 9 levels ($F_{(3, 8)} = 38.54$ $p <$ 0.0001) (E) PTN treatment did not show any change in pro-MMP 2 expression ($F_{(3, 8)} = 38.54$ $p = 0.0993$) (F)

PTN treatment significantly increased the MMP 2 levels ($F_{(3, 8)} = 6.154$ $p = 0.0179$). (B – C, E – F) Error bars represent standard error of mean (SEM), and symbols *, ** and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.



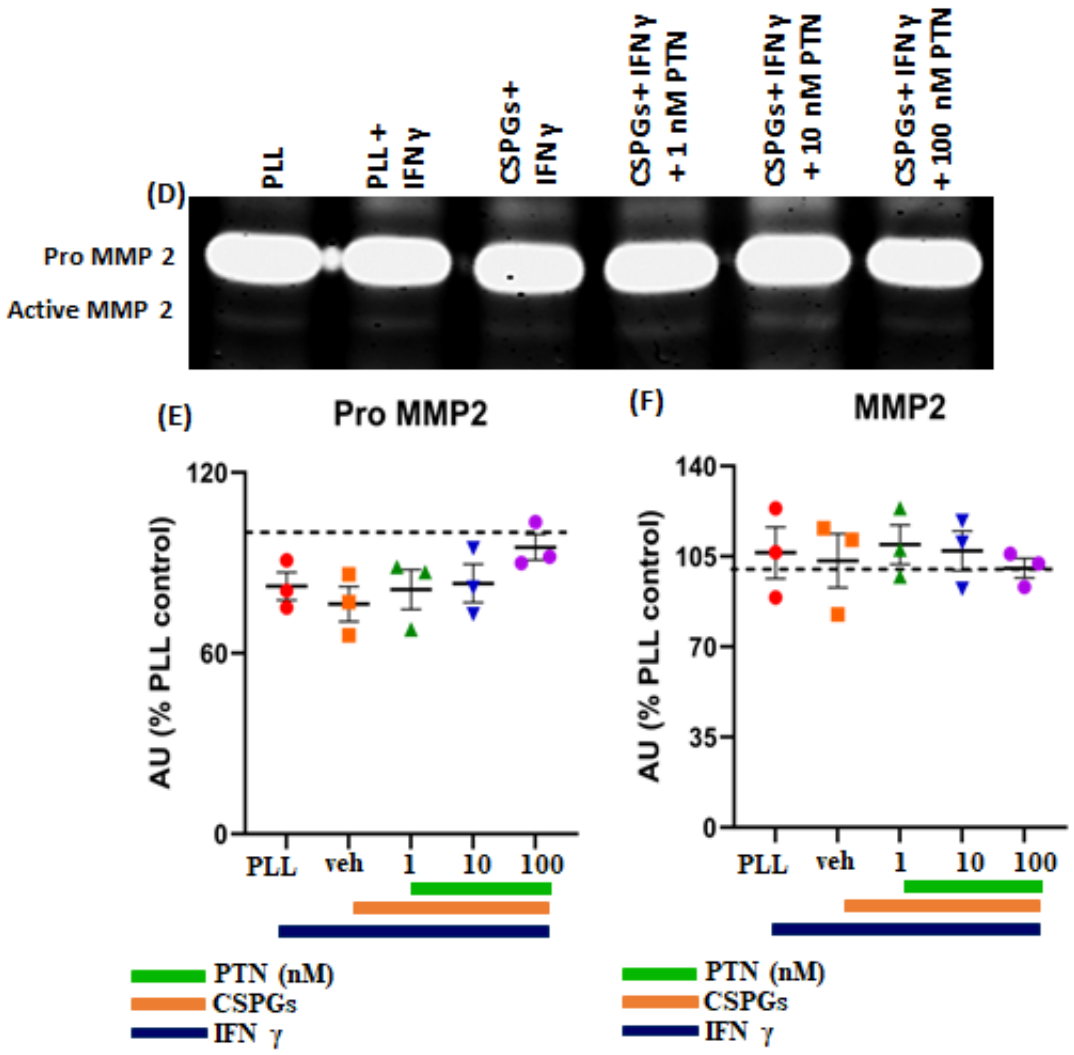


Figure 3.8: Effect of PTN treatment on abundance of MMP 9 and MMP 2 from IFN γ stimulated microglia

Activity of MMP 9 and MMP 2 were detected using gel zymography from conditioned media from IFN γ stimulated microglia treated with or without PTN. The intensities of the white band for (A) pro-MMP 9, active MMP 9 and (D) pro-MMP 2, active MMP 2 were calculated using Image J software. The intensities of PLL control were considered 100% for pro MMP 9, active MMP 9, Pro MMP 2 and active MMP 2. PTN significantly reduces the release of pro-MMP 9 from microglia ($F_{(4, 10)} = 58.32$ $P < 0.0001$). PTN did not show any difference in the released (C) active

MMP 9 ($F_{(4, 10)} = 0.9243$ $P = 0.4873$) (E) pro-MMP 2 ($F_{(4, 10)} = 1.539$ $P = 0.2640$) and (F) active MMP 2 ($F_{(4, 10)} = 0.1837$ $P = 0.9416$) levels. (B – C, E – F) Error bars represent the standard error of the mean (SEM), and symbols **** represent $P < 0.001$, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

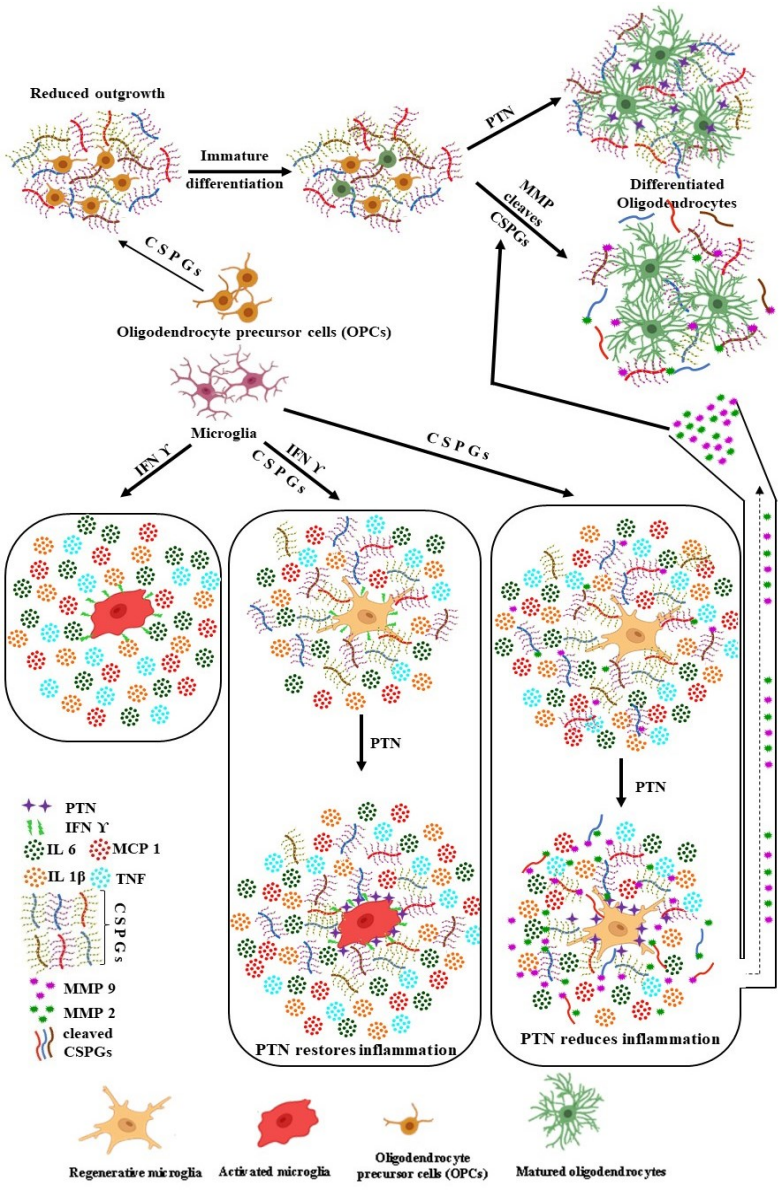


Figure 3.9: Summarized effect of PTN on OPCs and microglia in the presence of CSPGs

Microglia being the immune cells in the brain can be activated by the release of IFN γ, thus promoting the release of pro inflammatory cytokines. In the presence of CSPGs this pro inflammatory activated state is reduced and upon treatment with PTN the pro inflammatory activity is restored. On the contrary, in the absence of pro inflammatory stimulus the PTN reduced the expression of pro inflammatory cytokines and induced the expression of MMP9 and MMP 2. PTN also promoted the differentiation of OPCs to oligodendrocytes and MMPs released from microglia upon treatment with PTN can cleave CSPGs promoting the differentiation process.

Chapter 4: Pleiotrophin modulates the effect of CSPGs on the activity of astrocytes and mixed glia

Introduction

The brain consists of electrically active neurons whose function is coordinated by glial cells. Glial cells have distinct functions and interact with each other to maintain the normal physiological functioning of the central nervous system (CNS). Astrocytes and microglia are types of glial cells present in the brain, but have different origins. Microglia are derived from hematopoietic myeloid progenitor cells while astrocytes are derived from neuroepithelial progenitor cells [286, 287] . Microglia are highly motile cells, and the only immune cells of CNS that are associated with phagocytic activity [288].

Astrocytes communicate with microglia to create an environment favorable for neurons to function properly. Astrocytes play a significant role in establishing connections with blood vessels, forming end feet connections, and providing nutrients to neurons [38]. Astrocytes regulate synapse formation by releasing various factors like hevin, thrombospondin, glypican 4 and 6 [288]. Synaptic pruning is also an important process to eliminate extra synapses, and one of the methods by which microglia eliminate synapses is phagocytosis of the region with increased deposition of C1q, which happens due to astrocytic TGF β [288]. Astrocytes possess the capacity to express numerous ECM proteins necessary for constructing an extracellular matrix. One of the components of ECM are CSPGs, which function as guiding cues during neuronal development and regeneration. CSPGs direct the growth of axons, migration of cells, formation and stabilizing synapse formation [289]. Meanwhile, microglia can influence the extracellular matrix's composition, including the integrity of CSPGs, by releasing matrix metalloproteinases (MMPs) [170]. This dynamic interaction plays a role in shaping neurons' ability to establish stable connections[288].

During an injury in the brain or any neurological disease, astrocytes and microglia become activated by the cytokines released form damaged and dying cells surrounding the injury. These

activated glial cells can then upregulate the expression of chondroitin sulfate proteoglycans (CSPGs) [47] [216] [251]. Elevated CSPGs levels are known to inhibit the regeneration of neurons, inhibition of OPC proliferation and differentiation [158] [129]. Digestion of CSPGs with Chondroitinase ABC (ChABC) can enhance functional recovery following central nervous system (CNS) injuries [149]. However, cleaved components of CSPGs after ChABC digestion generate immunogenic stubs that can impair the regeneration process [290]. Moreover, owing to the polyanionic nature of CSPGs, they exhibit the capacity to ameliorate oxidative stress through the sequestration and complexation of active iron species within the proximate neuronal environment, consequently reducing the production of deleterious ROS. Additionally, CSPGs function as ionostatic agents, modulating the concentrations of charged ions such as sodium, potassium, and calcium, thereby contributing to ionic homeostasis and providing neuroprotection [178]. Identifying an approach to minimize the inhibition by CSPGs without affecting CSPG integrity could lead to better outcomes. In this study, we identify pleiotrophin (PTN) as a novel protein that can induce anti-inflammatory conditions even in the presence of CSPGs.

PTN is a developmentally regulated protein whose expression peaks during the early stage of CNS development and eventually decreases [291] [222]. The expression is increased during brain injury or any neurological disease [291]. PTN is known to potentiate the regeneration of neurons by overcoming the inhibition imposed by CSPGs [195]. PTN can induce differentiation of OPCs to oligodendrocytes [241]. PTN causes the release of neurotrophic factors from microglia [239].

All the available literature has shown the effect of CSPGs on neurons and microglia. However, few literatures are available that focus on the effect of PTN and CSPGs on astrocytes. PTN induces the release of pro-inflammatory cytokines in LPS-stimulated PTN Tg mice [270], but it is not clear whether this effect was due to the signaling of PTN in astrocytes or microglia. Thus,

in this study, we focused on determining the effect of PTN on astrocytes in the presence of CSPGs and the effect of PTN on mixed glia (astrocytes and microglia) in the presence of CSPGs.

We had two hypotheses: (1) PTN will reduce the pro-inflammatory activity of astrocytes in the presence of CSPGs, and (2) PTN will reduce the pro-inflammatory activity from mixed glia in the presence of CSPGs. To test the first hypothesis, astrocytes were isolated from mixed glia culture, prepared from 2-day-old CX3CR1/GFP mice pups. Astrocytes were cultured on a CSPGs matrix and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM) for 72 h, and proinflammatory cytokines released from different treatment conditions were measured.

To test the second hypothesis, mixed glia cultures were prepared from 2-day-old CX3CR1/GFP mice, cultured on CSPGs matrix, and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM). Proinflammatory cytokines released from different treatment conditions were measured.

Materials and Methods

Matrices preparation:

For astrocytes and mixed glia study, 12 mm coverslips (Fisherbrand, 12-545-81) were coated with 100 µg mL^{-1} Poly-L-Lysine (Sigma-Aldrich, P6282) for 2 hours at 37 °C and 5% CO_2. After incubation, the coverslips were washed with sterile distilled water, and the coverslips were coated with growth inhibitory matrix (1, 2.0, 2.5, and 5 µg mL^{-1}) of CSPGs (Sigma Aldrich, CC117) for 2 hours and washed with PBS before seeding cells on the coverslips.

Primary astrocytes cell culture:

All animal protocols were conducted in accordance with the Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the

University of Alberta. Mice primary astrocyte cell cultures were isolated from 2-day-old CX3CR1-GFP mice. The cortices were dissected and digested with TrypLE (Gibco, 12605-028) for 10 minutes at 37°C. The tissues were treated with 60 μg mL^{-1} of DNaseI (Sigma, DN25-100) for 5 min and centrifuged at 500 X g for 3 min. Cells were dissociated from the tissue by trituration in Dulbecco's modified Eagle's medium nutrient mixture F-12 (ham) (DMEM/F12) (Gibco, 11320-033) supplemented with 10% fetal bovine serum (Gibco, 12483-020) and 1% Penstrep (Gibco, 15140-122). Cells were then centrifuged and cultured in T 25 flasks coated with 100 μg mL^{-1} Poly-L-Lysine (Sigma-Aldrich, P6282) at 37 °C, 5% CO_2. 2/3 media was changed every 3 days; on day 9, the flasks were placed on a shaker at 50 rpm at 37 °C, 5% CO_2 to remove any loosely adherent cells. The media was changed, and flasks were placed on a shaker at 220 rpm for 16 hours at 37 °C, 5% CO_2 to detach OPCs and microglia. After incubation, the isolated OPCs and microglia were discarded, the remaining cells adhered to the flasks were washed vigorously with PBS to remove any other contaminated cells, and the remaining cells attached to the flasks cells were pure astrocytes. The astrocytes were then trypsinized and cultured in a T 75 flask (coated with 100 μg mL^{-1} poly L lysine) with media DMEM/F12 supplemented with 10 % fetal bovine serum (Gibco, 12483-020), 1 X GlutaMAX™ Supplement (Gibco, 35050079) and 1% Penstrep (Gibco, 15140-122). The cells were incubated at 37 °C, 5% CO_2 for 6 days. On 7^{th} day the cells were trypisinized and seeded at 1.5 X 10^5 cells / well onto coverslips coated with different matrices for 24 h in DMEM/F12, 1% GlutaMAX, 1% PS, and 1 % FBS at 37 °C, 5% CO_2. After incubation, the media was changed to media containing different concentrations of recombinant mouse PTN (R&D 6580-PL-050). The cells were incubated for 3 days at 37 °C, 5% CO_2. The media was collected and processed for cytokines and MMPs expression. Cells were then fixed with 5% formaldehyde for 20 minutes, washed with PBS, and processed for immunocytochemistry.

Primary mixed glia cell culture

Primary mixed glia cells were isolated from postnatal day 2 CX3CR1-GFP mice. The cortices were dissected and digested with TrypLE (Gibco, 12605-028) for 10 minutes at 37°C. The tissues were treated with 60 µg mL^{-1} of DNaseI (Sigma, DN25-100) for 5 min and centrifuged at 300 X g for 5 min. Cells were dissociated from the tissue by trituration in Dulbecco's modified Eagle's medium nutrient mixture F-12 (ham) (DMEM/F12) (Gibco, 11320-033) supplemented with 10% fetal bovine serum (Gibco, 12483-020) and 1% Penstrep (PS) (Gibco, 15140-122). Cells were centrifuged at 500 X g for 3 min and cultured in 12 well cell culture plates coated with 100 µg mL^{-1} Poly-L-Lysine (PLL) (Sigma-Aldrich, P6282) at 37 °C, 5% CO_2. Cells were cultured for 19 days at 37 ºC and 5% CO_2, with media changed twice weekly. On 20^{th} day for microglia isolation, confluent cell layers were washed with sterile PBS and treated with Trypsin-EDTA (0.25%) (Gibco, 25200072) and DMEM F12 mixture (1:3) for 20 minutes at 37 °C, 5% CO_2. The isolated cell layer consists of a mixed cell population, the cells were then centrifuged at 1200 rpm for 5 min and cultured on T 75 flask (coated with 100 µg mL^{-1} poly L lysine) for seven days with DMEM/F12, 1% GlutaMAX, 1% PS and 10 % FBS at 37 °C, 5% CO_2. The media was changed twice a week. After, 7 days of incubation, cells were trypsinized and seeded onto coverslips coated with different matrices for 24 h with DMEM/F12, 1% GlutaMAX, 1% PS, and 1 % FBS at 37 °C, 5% CO_2. After 24 h, the media was changed with media containing different concentrations of recombinant mouse PTN (R&D 6580-PL-050) for 72 h at 37 °C, 5% CO_2. The media was collected after 72 h and analyzed for cytokines and MMPs expression.

Cytokines analysis

The media collected from different treatment conditions were centrifuged at 2000 rpm for 10 min to remove any cell debris. The media was then analyzed for pro-inflammatory focused 10 plex discovery assay (Eve Technologies, Calgary, AB).

Gel zymography

Conditioned media from cell culture were centrifuged to remove cell debris and concentrated using an amicon ultra-2 centrifugal filter unit (Millipore UFC201024). MMP activity was determined by gel zymography using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with modification. The samples were run on 7.5% acrylamide gel with 0.1% (w/v) gelatin. 15 ug protein was loaded into each well, diluting 5 X sample buffer to 1 X buffer and run under non-reducing conditions at 110 V and 4 °C for 2 h. After electrophoresis, gels were washed with wash buffer (2.5 % Triton X 100, 50 mM Tris HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 2 X 30 min with agitation. The gels were then incubated in incubation buffer (1.0 % Triton X 100, 50 mM Tris HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 24 h at 37 °C. The gels were then stained with 0.5% Coomassie blue (0.5% Coomassie blue, 50% water, 10% acetic acid, 40 % methanol) for 1 h. The gels were then destained with destaining solution (40% methanol, 10% acetic acid, 50% water) until white bands were detected under a blue background. Gels were then imaged using a Licor Odyssey reader. The bands were analyzed using a gel analyzer plugin in Fiji [230].

Immunocytochemistry

The fixed samples were blocked with 10% normal horse serum in PBS containing 0.1% Tritton-X 100 and washed with PBS. Samples were stained with glial fibrillary acid protein (GFAP) (1:500, abcam, ab68428) in PBS pH 7.4 with 0.1% HS, overnight at 4° C. The primary antibody GFAP was detected with the secondary antibody donkey anti-mouse Alexa 647 (1:500

invitrogen A-31573). The samples were then mounted on slides with fluormount-G (Southern Biotech). The images were taken using a Leica TCS-SPE fluorescent microscope.

Statistics

Statistical analyses of data from fluorescent images were carried out using one-way ANOVA followed by Tukey's multiple comparisons test for significance between treatment groups. For SHOLL analysis, the data were analyzed using one-way ANOVA and Tukey's multiple comparisons test for significance between treatment groups. n represents a single independent experiment (i.e., an independent culture preparation) with a minimum of three technical replicates. Each technical replicate represents a well in a 24-well culture plate. All statistical analyses were done using Graphpad Prism version 9.1.2.

Results

Effect of CSPGs on morphology of astrocytes

To determine the effect of PTN on astrocytes in the presence of CSPGs, we first identified the purity of astrocytes in cell culture by performing immunostaining for GFAP and found that cells were 90 – 95 % pure astrocytes, with other cells primarily microglia (Figure 4.1 A – C). To verify adhesion of astrocytes to CSPGs matrix, immunofluorescence staining with GFAP was done on astrocytes cultured for 96 h on coverslips coated with PLL (Figure 4.2 A – C), 1 µg mL^{-1} CSPGs (Figure 4.2 D – F), 2.5 µg mL^{-1} CSPGs (Figure 4.2 G – I) and 5 µg mL^{-1} CSPGs (Figure 4.2 J – L). The astrocytes remained attached to the matrix even at a higher concentration of 5 µg mL^{-1} of CSPGs, but the number of astrocytes were greatly reduced. Fluorescence microscopy showed that in the presence of CSPGs astrocytes acquire different morphology with hypertrophic cell bodies and reduced number of projections in a dose-dependent manner with very few projections at higher concentrations of 5 µg mL^{-1} CSPGs (Figure 4.2 K – L). In the presence of CSPGs astrocytes form

clusters indicated with yellow arrows (Figure 4.2 I, F, and L). Thus, based on this data for further experiments to determine the effect of PTN on astrocytes in the presence of CSPGs, 2.0 µg mL^{-1} CSPGs concentration was decided for the matrix preparation as at this concentration astrocytes remain adhere to the matrix and show activated astrocyte morphology.

Effect of PTN on the release of pro-inflammatory cytokines from astrocytes in the presence of CSPGs

Released pro-inflammatory cytokines were measured to determine the effect of PTN on astrocytes in the presence of CSPGs. Isolated astrocytes were cultured on a CSPGs matrix (2.0 µg mL^{-1}) and treated with different concentrations of PTN (1 nM, 10 nM, 100 nM PTN) for 72 h. The media was collected and processed to detect the following cytokines (IL 6, TNF, and MCP 1). The data indicates that CSPGs do not affect the release of TNF and IL 6, but with the presence of PTN, the expression of the TNF, IL 6, and MCP 1 was increased. In the presence of CSPGs, PTN treatment significantly increased the expression of TNF cytokine ($F_{(3, 8)} = 204.7$ $P < 0.0001$); and post hoc analysis further showed that with PTN treatment the expression was significantly increased at higher concentration of 10 nM PTN ($P < 0.0001$) and 100 nM PTN ($P < 0.0001$), though 1 nM PTN did not induce any change in expression of TNF ($p = 0.9529$) (Figure 4.3 A).

CSPGs did not induce any change in the expression of IL 6, and with PTN treatment the expression of IL 6 was significantly increased ($F_{(3, 8)} = 248.0$ $P < 0.0001$). Post hoc analysis suggests that PTN at a lower concentration of 1 nM did not induce the release of IL 6 ($P = 0.6512$), but the expression was increased at a higher concentrations of 10 nM PTN ($P < 0.0001$) and with 100 nM PTN ($P = 0.0010$). The expression of IL 6 was significantly higher at 10 nM PTN compared to 1 nM PTN ($P < 0.0001$) and 10 nM PTN ($p < 0.0001$) (Figure 4.3 B). Here, CSPGs reduced the release of MCP 1 and PTN treatment significant increased the release of MCP 1 ($F_{(3,}$

$_{8)}$ = 539.9 $P < 0.0001$). Similarly, to TNF and IL 6; PTN at 1 nM concentration did not show any difference in the release of MCP 1, while post hoc analysis suggests that higher concentrations of 10 nM and 100 nM PTN significantly increased the expression of MCP 1 ($P < 0.0001$), also the expression was highest at 10 nM PTN treatment (Figure 4.3 C). Thus, the data generated suggests that in the presence of CSPGs, PTN induces the release of pro inflammatory cytokines (IL 6, TNF and MCP 1) from astrocytes.

Effect of PTN on the release of MMP from astrocytes in the presence of CSPGs

We next determined the expression of MMPs from astrocytes. Astrocytes play a major role in maintaining the extracellular milieu by releasing various extracellular components and matrix metalloproteinase (MMP) that shape the extracellular matrix[29247]. To determine the release of MMPs from astrocytes, the conditioned media was collected and concentrated using ampicon. The concentrated media was then subjected to SDS PAGE under non-reducing conditions in gels with 0.1% (w/v) gelatin. The gels were then incubated to determine the proteolytic activity of MMP 9 and MMP 2. The gels were stained with Coomassie Brilliant Blue, and white bands corresponding to MMP 9 and MMP 2 molecular weight were observed in a dark background (Figure 4.4 A, D). In the presence of CSPGs , astrocytes release a higher amount of pro-MMP 9 compared to PLL control (512.52 %) (Figure 4.4 B), with PTN treatment, there was a significant increase in the release of pro MMP 9 ($F_{(3,8)} = 18.93$ $P < 0.0001$), and post hoc analysis showed that 10 nM PTN concentration significantly increased the expression ($P = 0.0030$), while 1 nM and 10 nM PTN did not show any increase in the release of pro MMP 9 (Figure 4.4 B). Similar to the pro MMP 9 release, CSPGs also increased the amount of MMP 9 expression by 126.75 % of PLL control (Figure 4.4 C). Contrary to the effect of PTN on the expression of pro-MMP 9, PTN significantly reduced the expression of MMP 9 ($F_{(3, 8)} = 16.14$ $P = 0.0009$) in a dose-dependent manner, and

post hoc analysis showed that 100 nM PTN concentration significantly reduced MMP 9 expressions ($P = 0.0008$). There was no change in the expression of pro-MMP 2 release compared to PLL control ($F_{(3, 8)} = 0.2165$ $P = 0.8823$) (Figure 4.4 E). There was a trend of reduced expression of MMP 2 with the PTN treatment but it was not significant conditions ($F_{(3, 8)} = 2.696$ $P = 0.1166$) (Figure 4.4 F). Thus, the data suggests that in the presence of CSPGs, PTN treatment increases the level of pro MMP 9 at 10 nM PTN and decreases the levels of MMP 9, while there was no difference in the level of pro MMP 2 and MMP 2 from astrocytes.

Effect of PTN on the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs

To assess the effect of PTN on mixed glia in the presence of CSPGs, mixed glia were cultured on CSPGs matrix (2.0 μg mL^{-1}) and treated with different concentrations of PTN (1nM, 10 nM, and 100 nM) for 72 h. The media was then assessed for released pro-inflammatory cytokines. There was a significant main effect of treatment on the release of IL 6 ($F_{(3, 8)} = 24.66$ $P = 0.0002$), and post hoc analysis showed that 1 nM PTN significantly increased the release of IL 6 compared to 10 nM PTN ($P = 0.0004$) and 100 nM PTN ($P = 0.0004$) (Figure 4.5 A). A main effect of treatment was also observed for TNF ($F_{(3, 8)} = 20.54$ $P = 0.0004$), and post hoc analysis also suggests that 1 nM PTN increases the expression of TNF compared to 10 nM PTN ($P = 0.0014$) and 100 nM PTN ($P = 0.0007$) (Figure 4.5 B). Similarly, treatment exhibited a main effect on the release of MCP 1 ($F_{(3, 8)} = 15.14$ $P = 0.0012$), and post hoc analysis suggests that 1 nM PTN significantly induced release of MCP 1 compared to 10 nM PTN ($P = 0.0025$) and 100 nM PTN ($P = 0.0019$) (Figure 4.5 C). Astrocytes and microglia upon stimulation with LPS release IL 12p70 [293]. Here, IL 12p70 release exhibited a significant treatment effect ($F_{(3, 8)} = 6.916$ $P = 0.013$), and post hoc analysis suggested that 10 nM PTN there was a significant release of IL 12p70 ($P =$

0.0179) (Figure 4.5 D). The data suggest that in the presence of CSPGs, PTN treatment induces the expression of pro inflammatory cytokines (IL 6, TNF, and MCP 1) at lower concentration (1 nM) and reduces at higher concentration (10 nM and 100 nM PTN) from mixed glia. The expression of IL 12 p70 cytokine with PTN treatment from mixed glia in the presence of CSPGs showed an maximum expression at 10 nM PTN and its expression was reduced at 100 nM PTN.

Effect of PTN on the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs during inflammatory stimulus

To assess the effect of PTN during inflammatory stimulus on mixed glia in the presence of CSPGs, mixed glia were cultured on CSPGs matrix and treated with different concentrations of PTN (1 nM, 10 nM, and 100 nM) with 100 ng mL^{-1} IFN γ for 72 h. The media was then assessed for released pro-inflammatory cytokines. During an inflammatory stimulus, in the presence of CSPGs significantly reduce the release of TNF ($F_{(4, 10)} = 50.40$ $P < 0.0001$), and post hoc analysis suggests that 10 nM PTN significantly increases the release of TNF ($P = 0.042$) (Figure 4.6 A), the expression of IL 6 was increased and showed a significant main treatment effect on the release of IL 6 ($F_{(4, 10)} = 28.78$ $P < 0.0001$) (Figure 4.6 B), post hoc analysis did not show any difference with PTN treatment. There was a treatment effect on MCP 1 release ($F_{(4, 10)} = 5.029$ $P = 0.0175$) but post hoc analysis did not show any difference with PTN treatment (Figure 4.6 C). However, CSPGs increased the expression of IL12p70 but did not show any difference with the PTN treatment ($F_{(4, 10)} = 2.748$ $P = 0.0887$) (Figure 4.6 D). The data suggests that in the presence of inflammatory stimulus of IFN γ, CSPGs reduces the expression of pro inflammatory cytokines (TN, IL 6 and MCP 1) and with PTN treatment there was a trend in increasing the expression of pro inflammatory cytokines (TNF, IL 6, MCP 1 and IL 12 p70) with increasing concentration but there was no significant difference.

Effect of PTN on the expression of MMPs from mixed glia in the presence of CSPGs

The media was collected to assess the effect of MMPs released from mixed glia, and the activity of MMPs was assessed through gel zymography (Figure 4.7 A – D). A main treatment effect on the release of pro MMP 9 was observed ($F_{(3, 8)} = 29.37$ $P = 0.0001$), and post hoc analysis showed that there was a significant dose dependent decrease in the levels of pro MMP 9 with PTN treatment with maximum decrease at 100 nM PTN concentration (Figure 4.7 B). There was no significant treatment effect on the release of MMP 9 ($F_{(3, 8)} = 0.8371$ $P = 0.5105$). Similarly, treatment did not show any treatment effect on the release of Pro MMP 2 ($F_{(3, 8)} = 1.407$ $P = 0.3101$) and MMP 2 ($F_{(3, 8)} = 0.1344$ $P = 0.9368$).

During an inflammatory stimulation with IFN γ, there was a main effect on the expression of pro MMP9 ($F_{(4, 10)} = 46.41$ $P < 0.0001$), but post hoc did not show reveal any significant difference (Figure 4.8 A - B) or MMP9 ($F_{(4, 10)} = 1.868$ $P = 0.1928$) (Figure 4.8 C), pro-MMP 2 ($F_{(4, 10)} = 0.1984$ $P = 0.9335$), or MMP 2 ($F_{(4, 10)} = 0.3584$ $P = 0.8326$) (Figure 4.8 F). Thus, the data suggest that with PTN treatment the expression of pro MMP 9 is reduced in mixed glia while there was no effect on the levels of MMP9, pro MMP 2, or MMP 2.

Discussion

PTN activates astrocytes and induces the release of pro-inflammatory cytokines

Following CNS injury or any neurological diseases, glial cells become activated and release cytokines and growth factors that can positively or negatively affect the regeneration process [29429547296]. Activated astrocytes release CSPGs, which are major component of glial scar and are known to inhibit the regeneration of neuronal growth [47]. PTN is upregulated during CNS injury and CNS diseases [270]. One of the major findings of this study identified the effect of PTN on astrocytes in the presence of CSPGs. The data suggests that with 2.0 µg mL^{-1} CSPGs matrix,

astrocytes morphology is altered with reduced process outgrowth [297]. Although CSPG matrices induced a slight increase in the release of IL 6 and TNF compared to the control, with PTN treatment there was a significant increase in the release of IL 6 and TNF at higher concentrations (10 nM and 100 nM PTN). Similarly, although CSPGs reduced the expression of MCP 1, with PTN treatment there was an increase in the expression of MCP 1 at higher concentrations. Previously available literature indicates that PTN reduces the pro-inflammatory effect of LPS on astrocytes [270]. Moreover, astrocyte-derived PTN is associated with anti-inflammatory and neuroprotective role in EAE mouse model [298]. Thus, our data shows that PTN can induce the release of pro-inflammatory cytokines from astrocytes in the presence of CSPGs.

PTN modulates the expression of pro-inflammatory cytokines from mixed glia in the presence of CSPGs.

Microglia and astrocytes are essential glial cells within the CNS that communicate with each other to synchronize their functions and establish communication with neurons to provide crucial support for the proper development and functioning of the central nervous system (CNS). Microglia and astrocytes communicate with each other by cytokines [288299]. Various studies have identified the direct effect on astrocyte derived cytokines on microglia activity [288]. In the previous chapter, we found that PTN reduces the expression of pro-inflammatory cytokines from microglia in the presence of CSPGs. Contrary to the effect of PTN on microglia in the presence of CSPGs, PTN treatment induces the expression of pro-inflammatory cytokines from astrocytes in the presence of CSPGs. To provide a more comprehensive assessment of the effect of PTN on microglia and astrocytes, mixed glia were cultured on CSPGs matrix and treated with PTN. The data generated from this study indicates that when mixed glia were treated with PTN in the presence of CSPGs the expression of pro-inflammatory cytokines IL 6, TNF, and MCP 1 was

reduced, which correlates to the PTN effect on isolated microglia. Previous studies have shown that activated astrocytes and microglia release IL 12 p70 upon stimulation with LPS [293]. Here in the presence of CSPGs, mixed glia released IL 12 p70 and upon treatment with PTN the expression was increased with 10 nM PTN and reduced at 100 nM PTN. Thus, the data generated from this study indicates that at higher concentrations of PTN (10 nM and 100 nM), there is a significant decrease in the expression of pro-inflammatory cytokines, except IL 12 p70 whose expression peaked at 10 nM PTN but reduced at 100 nM PTN from mixed glia in the presence of CSPGs.

In this study, we also simulated an initial inflammatory state following an injury by activating mixed glia using IFN γ [283,284]. In mixed glia activated with IFN γ, there was no effect of PTN treatment on the release of pro-inflammatory cytokines IL 6, TNF, MCP 1, and IL 12 p70 in the presence of CSPGs. Previous literature has shown that astrocytes and microglia have the opposite response to IFN γ signaling [300]. Similarly, our studies found that CSPGs reduce pro-inflammatory cytokines in microglia and increase expression in astrocytes. Moreover, upon treatment with PTN, the response of CSPGs is enhanced. Due to this opposite effect of PTN on astrocytes and microglia, in a mixed glia environment the magnitude of response was less compared to individual cell types, and skewed towards the microglial effects.

PTN reduces the release of MMPs in the presence of CSPGs

Pleiotrophin treatment modulates the cytokines released from the cells and affects the release of MMPs released from cells. In the brain, astrocytes are one of the major cell types in brain that release components for ECM [30147]. During injury in CNS or neurological disease, the levels of MMP 9 and MMP 2 are increased [301]. Here, CSPGs increased the expression of pro-MMP 9, and with PTN treatment there was a dose-dependent effect in the released levels of pro-MMMP 9, with 10 nM PTN showing an optimal release. However, the active MMP 9 level decreased with

increasing PTN concentration. Although there was no significant effect on the release of pro-MMP 2 with PTN treatment in the presence of CSPGs, there was a decreasing trend in the levels of active MMP 2.

The levels of MMP-9, an enzyme crucial for tissue remodeling, can be influenced by various factors. One potential mechanism involves the conversion of inactive pro-MMP-9 to its active form by MMP-2 and MMP-13 enzymes, as well as the regulation by tissue inhibitor of MMPs (TIMPs) [302]. TIMPs inhibits the activity of MMPs either by directly binding to active site of MMPs, forming complex with active MMPs and chelating zinc ion required for MMPs activation [303]. Notably, IL 1 β and TNF are known activator for the release of TIMPs from astrocytes [304].

In the context of the data generated in this chapter, it is evident that when CSPGs are present, PTN has the effect of triggering the release of TNF from astrocytes. This TNF release has the potential to stimulate the release of TIMPs from astrocytes. As a result, this could lead to a reduction in the activity of MMP-9, an enzyme responsible for matrix degradation. Additionally, the diminished expression of MMP2 also contributes to a decrease in the conversion of pro-MMP-9 to its active form, MMP-9.

In mixed glia conditions although there was an overall decrease in the levels of pro MMP 9 suggesting that PTN treatment reduces the expression of MMP 9 along with reduced conversion to active MMP 9 due to TIMP released form astrocytes. This study shows the complex role of PTN in regulating the expression of inflammatory factors and MMPs from astrocytes and mixed glia in the presence of CSPGs.

Conclusion

Chapter 4 investigated the effect of PTN on astrocytes and missed glia in the presence of CSPGs under non-inflammatory and inflammatory conditions. Our results suggest that PTN can

modulate the activity of astrocytes and microglia to favour regeneration after an injury, but that the response depends upon the activated phenotype and the cellular milieu. PTN induces the release of pro-inflammatory cytokines from astrocytes in the presence of CSPGs, but in mixed glia conditions there is a reduction in the release of pro-inflammatory cytokines. Under inflammatory stimulation mimicking the early stage of injury, PTN can enhance the inflammatory stimulus to augment inflammatory processes that may be beneficial during early stage of injury.

Figures

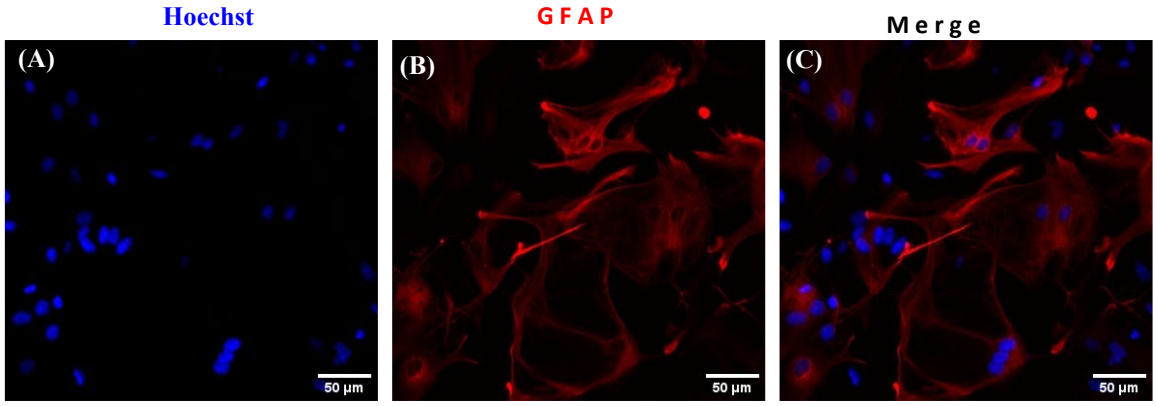


Figure 4.1: Purity of astrocytes by immunofluorescence

Isolated astrocytes were seeded on poly L lysine matrix (PLL). Fixed cells were stained with (A) Hoechst for nuclear stain and (B) astrocytes with anti-GFAP antibody (C) merge. Epifluorescent images of astrocytes were acquired at 20 X. The scale bar represents 50 µm.

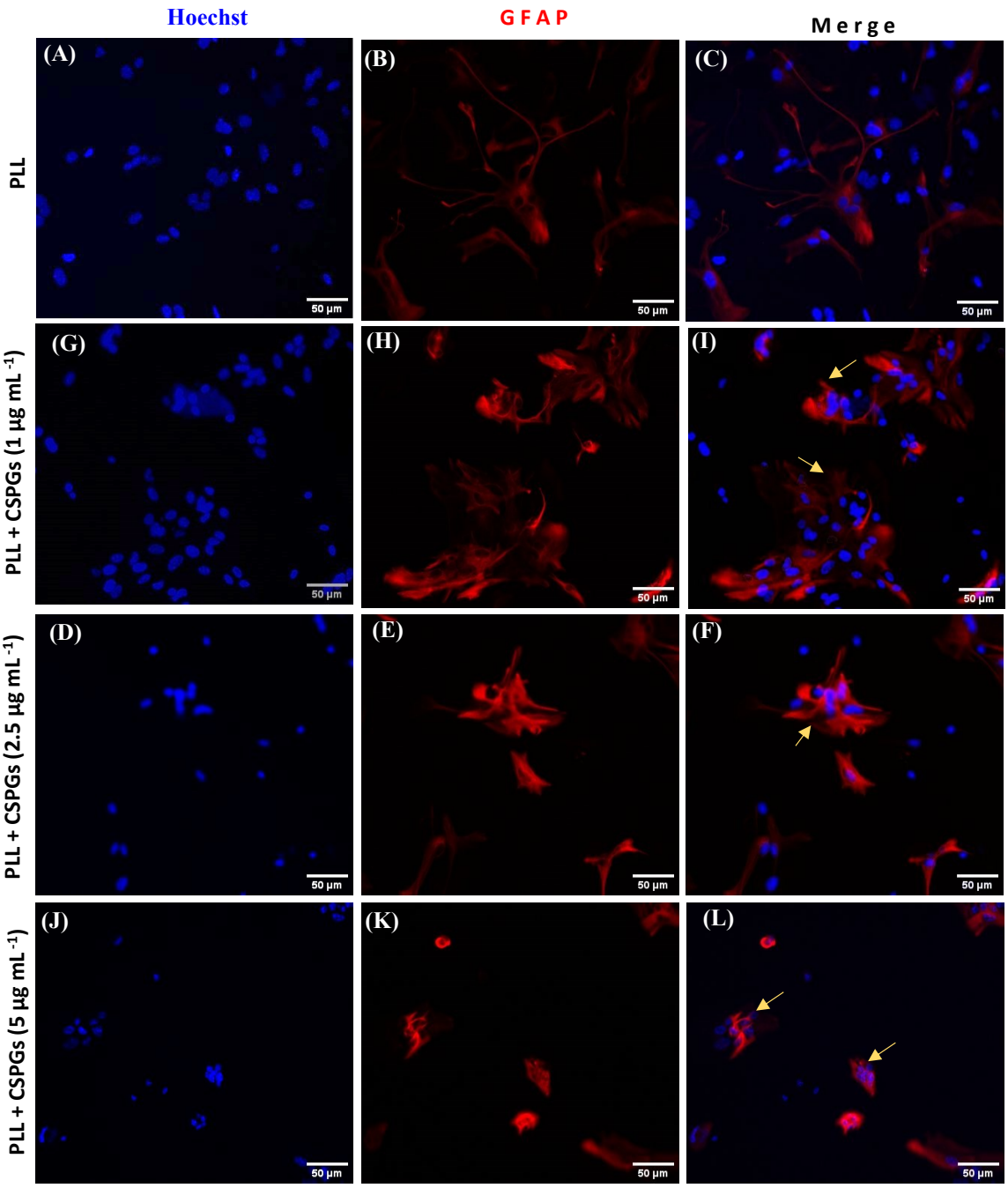


Figure 4.2: Astrocytes cultured on CSPGs matrix.

Isolated astrocytes were seeded on poly L lysine (PLL) matrix and PLL + CSPGs (different concentrations) for 72 h. Fixed cells were immunostained with anti-GFAP and Hoechst and imaged at 20 X under an epifluorescent microscope. Yellow arrow indicating clumped astrocytes

with reduced outgrowth. (A – C) astrocytes cultured on PLL matrix, (D – F) astrocytes cultured on PLL + CSPGs (1 μg mL $^{-1}$), (G – I) astrocytes cultured on PLL + CSPGs (2.5 μg mL $^{-1}$), (J – L) astrocytes cultured on PLL + CSPGs (5 μg mL $^{-1}$). The scale bar represents 50 μm.

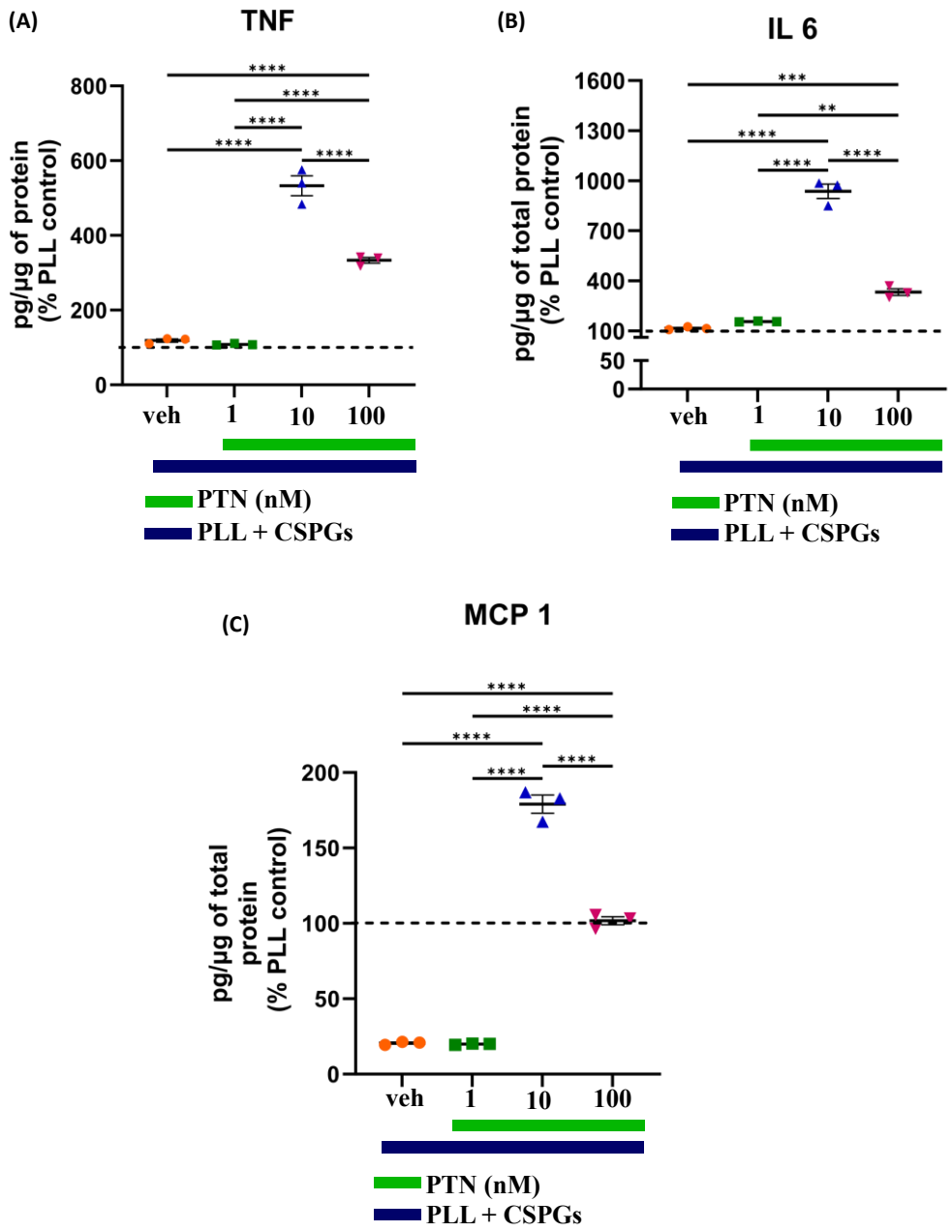
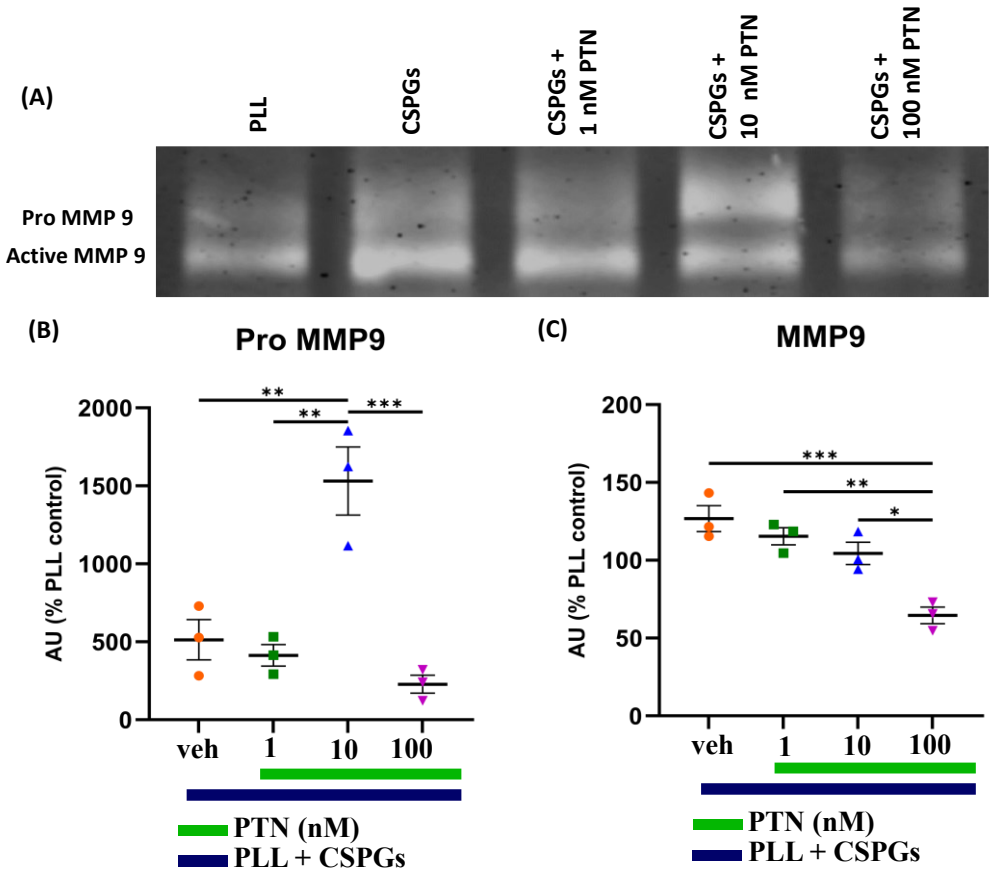


Figure 4.3: PTN treatment induced the release of pro-inflammatory cytokines from astrocytes.

Astrocytes were cultured on CSPGs matrix and treated with different concentrations of PTN. PTN significantly increases the expression of (A) TNF ($F_{(3, 8)} = 204.7$ $p < 0.0001$), (C) IL 6 ($F_{(3, 8)} =$

248.00 $p < 0.0001$), (D) MCP 1 ($F_{(3, 8)} = 539.9$ $p < 0.0001$). (A – C) Error bars represent the standard error of the mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats. All data are based on 3 independent experiments with a minimum of three technical replicates.


(A)
PLL
CSPGs
CSPGs + 1 nM PTN
CSPGs + 10 nM PTN
CSPGs + 100 nM PTN
Pro MMP 9
Active MMP 9
(B)
Pro MMP9
AU (% PLL control)
2000
1500
1000
500
0
**
**
***
veh
1
10
100
PTN (nM)
PLL + CSPGs
(C)
MMP9
AU (% PLL control)
200
150
100
50
0
***
**
*
veh
1
10
100
PTN (nM)
PLL + CSPGs

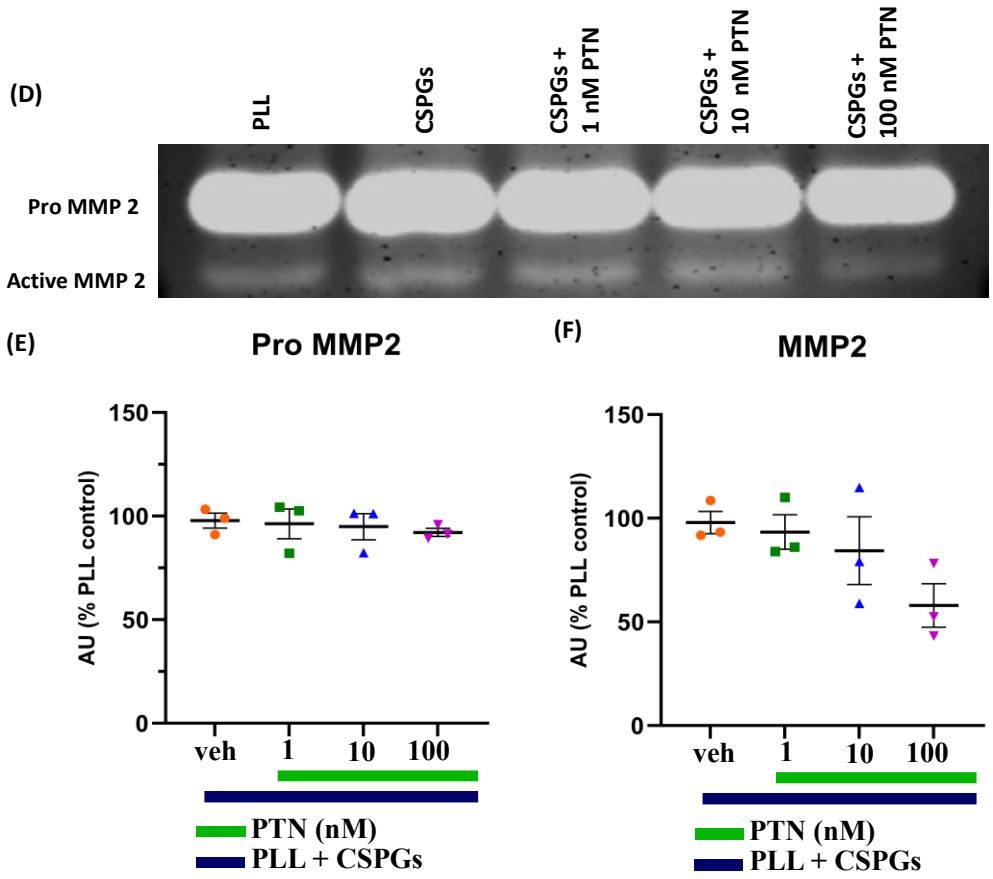


Figure 4.4 PTN treatment reduced the release of MMPs from astrocytes in the presence of CSPGs.

MMP 9 and MMP 2 activity was detected using gel zymography from conditioned media from astrocytes treated with or without PTN. The intensities of the white band for (A) pro-MMP 9, active MMP 9, and (D) pro-MMP 2, active MMP 2 were calculated using image J software. The intensities of PLL control were considered 100 % for pro-MMP 9, active MMP 9, Pro MMP 2, and active MMP 2. (B) PTN significantly reduces the release of pro-MMP 9 from microglia ($F_{(3, 8)} = 18.93$ $p = 0.0005$) (C) PTN significantly decreased active MMP 9 levels ($F_{(3, 8)} = 16.14$ $p = 0.0009$) (E) PTN treatment did not show any change in pro-MMP 2 expression ($F_{(3, 8)} = 0.2165$ $p = 0.8823$) (F) PTN treatment did not show any difference in the MMP 2 levels ($F_{(3, 8)} = 2.696$ $p =$

0.1166). (B – C, E – F) Error bars represent standard error of mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

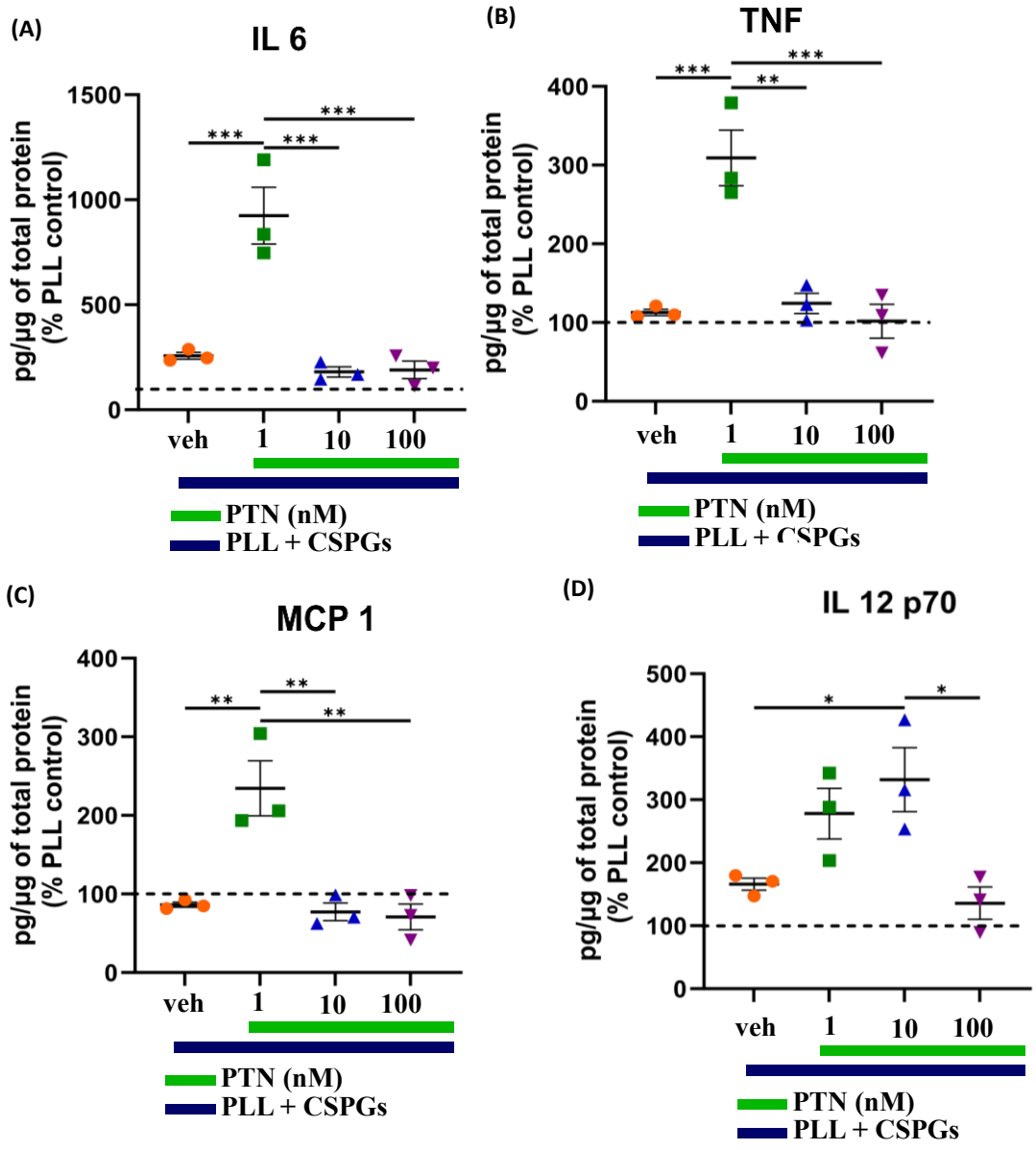


Figure 4.5: PTN treatment reduced the release of pro-inflammatory cytokines from mixed glia.

Mixed glia were cultured on a CSPGs matrix and incubated with different concentrations of PTN for 72 h; the conditioned media was collected and assessed for pro-inflammatory cytokine release. PTN significantly decreased the expression of (A) IL 6 ($F_{(3, 8)} = 24.66$ $p = 0.0002$), (B) TNF ($F_{(3,}$

$_{8)}$ = 20.54 p = 0.0004), (C) MCP 1 ($F_{(3, 8)}$ = 15.14 p = 0.0012) and (D) IL 12 p70 ($F_{(3, 8)}$ = 6.916 p = 0.013) at 10 nM and 100 nM PTN. (A – D) Error bars represent the standard error of the mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

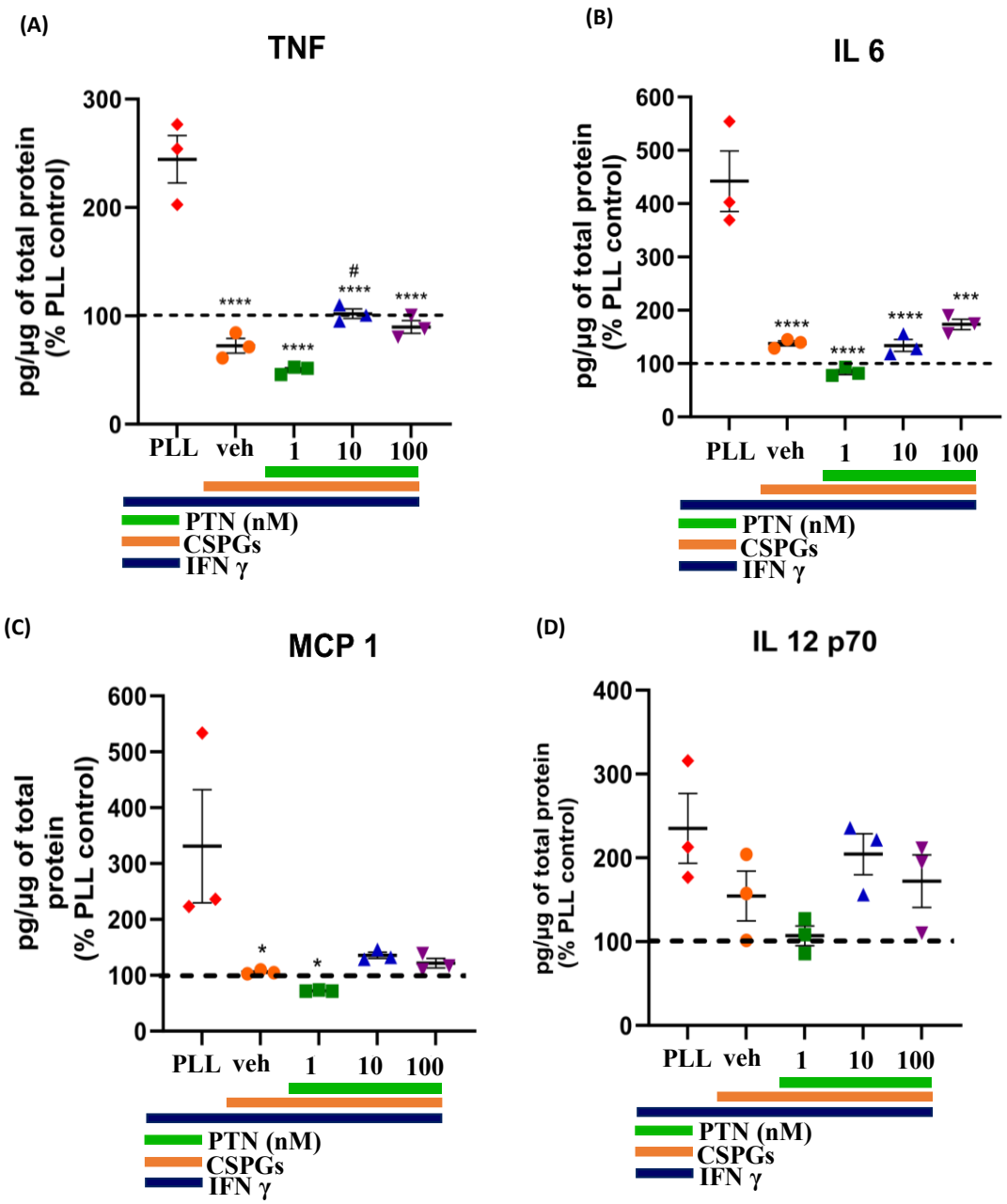
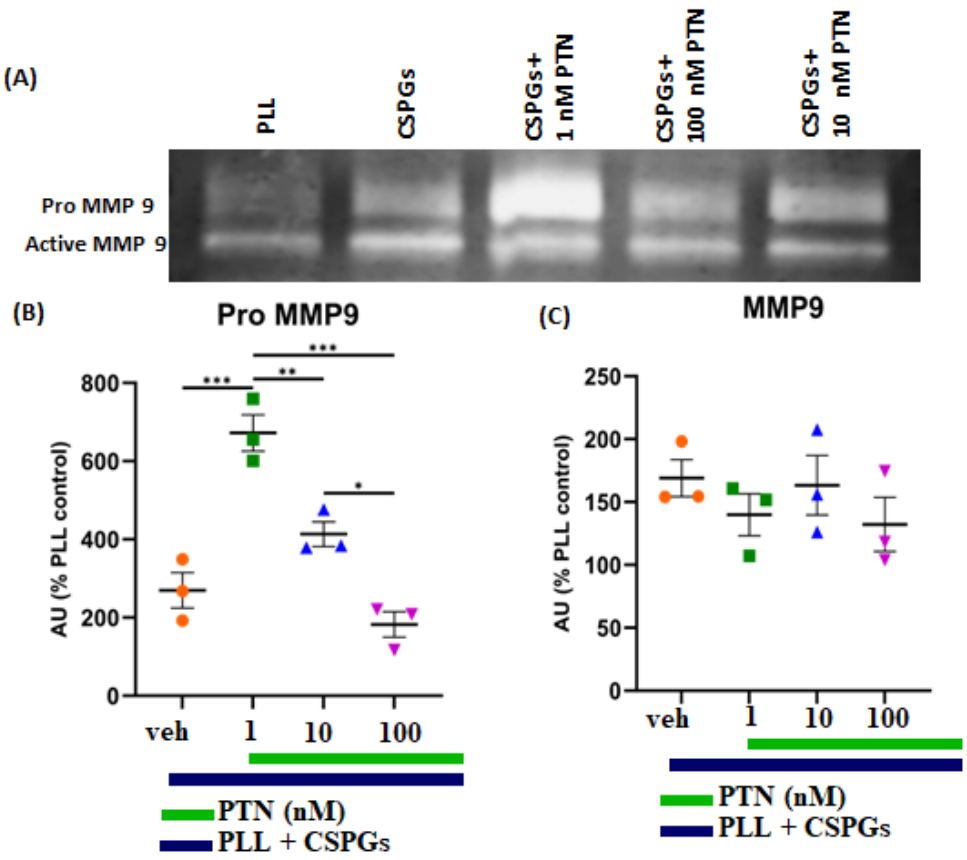


Figure 4.6: Effect of PTN treatment on the release of pro-inflammatory cytokines from mixed glia under inflammatory stimulus

Mixed glia were cultured on a CSPGs matrix and incubated with different concentrations of PTN for 72 h, the conditioned media was collected and assessed for pro-inflammatory cytokine release. PTN treatment did not alter the expression of (A) TNF ($F_{(4, 10)} = 28.78$ $p < 0.0001$), (B) IL 6 ($F_{(4,}$

$_{10)}$ = 20.54 p = 0.0004), (C) MCP 1 ($F_{(4, 10)}$ = 5.029 p = 0.0175) and (D) IL 12 p70 ($F_{(4,10)}$ = 2.748 p = 0.0887). (A – D) Error bars represent the standard error of the mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. " * " – compared to PLL + IFN γ, " # " – compared to 1 nM PTN control. All data are based on 3 independent experiments with a minimum of 3 technical repeats.


(A)
PLL
CSPGs
CSPGs+ 1 nM PTN
CSPGs+ 100 nM PTN
CSPGs+ 10 nM PTN
Pro MMP 9
Active MMP 9
(B)
Pro MMP9
AU (% PLL control)
800
600
400
200
0
***
**
***
*
veh
1
10
100
PTN (nM)
PLL + CSPGs
(C)
MMP9
AU (% PLL control)
250
200
150
100
50
0
veh
1
10
100
PTN (nM)
PLL + CSPGs

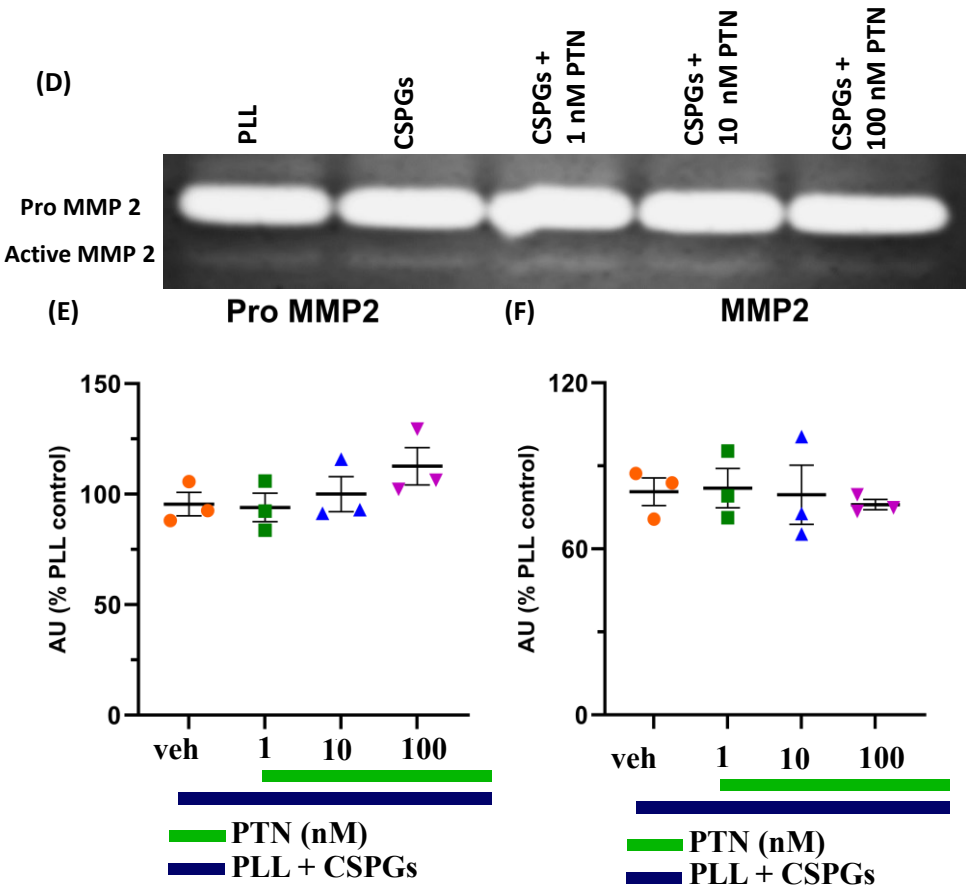
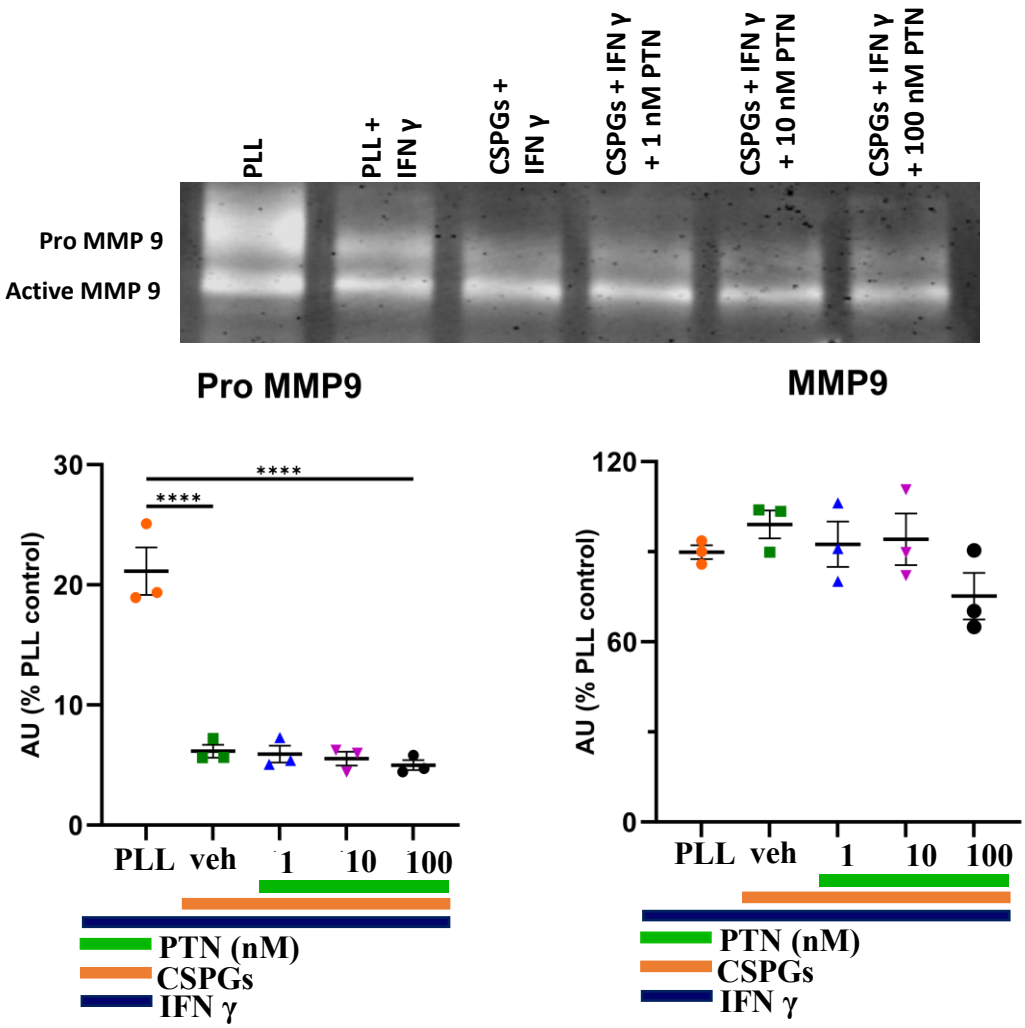


Figure 4.7: PTN treatment reduced the release of MMP 9 from mixed glia.

MMP 9 and MMP 2 activity was detected using gel zymography from conditioned media from microglia treated with or without PTN. The intensities of the white band for (A) pro-MMP 9, active MMP 9, and (D) pro-MMP 2, active MMP 2 were calculated using image J software. The intensities of PLL control were considered 100 % for pro-MMP 9, active MMP 9, Pro MMP 2, and active MMP 2. (B) PTN significantly reduces the release of pro-MMP 9 from mixed glia cultures ($F_{(3, 8)} = 29.37$ $p = 0.0001$) (C) PTN do not induce any change in MMP 9 levels ($F_{(3, 8)} = 0.8371$ $p = 0.5105$) (E) PTN treatment did not show any change in pro-MMP 2 levels ($F_{(3, 8)} = 1.407$ $p = 0.3101$), (F) PTN treatment do not alter the expression of active MMP 2 ($F_{(3, 8)} = 0.1344$ $p = 0.9368$). (B – C, E – F) Error bars represent standard

error of mean (SEM), and symbols *, **, and ***/**** represent $p < 0.05$, 0.01, and 0.001, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.


PLL
PLL + IFN γ
CSPGs + IFN γ
CSPGs + IFN γ + 1 nM PTN
CSPGs + IFN γ + 10 nM PTN
CSPGs + IFN γ + 100 nM PTN
Pro MMP 9
Active MMP 9
Pro MMP9
MMP9
AU (% PLL control)
30
20
10
0
****
****
PLL
veh
1
10
100
PTN (nM)
CSPGs
IFN γ
120
60
0

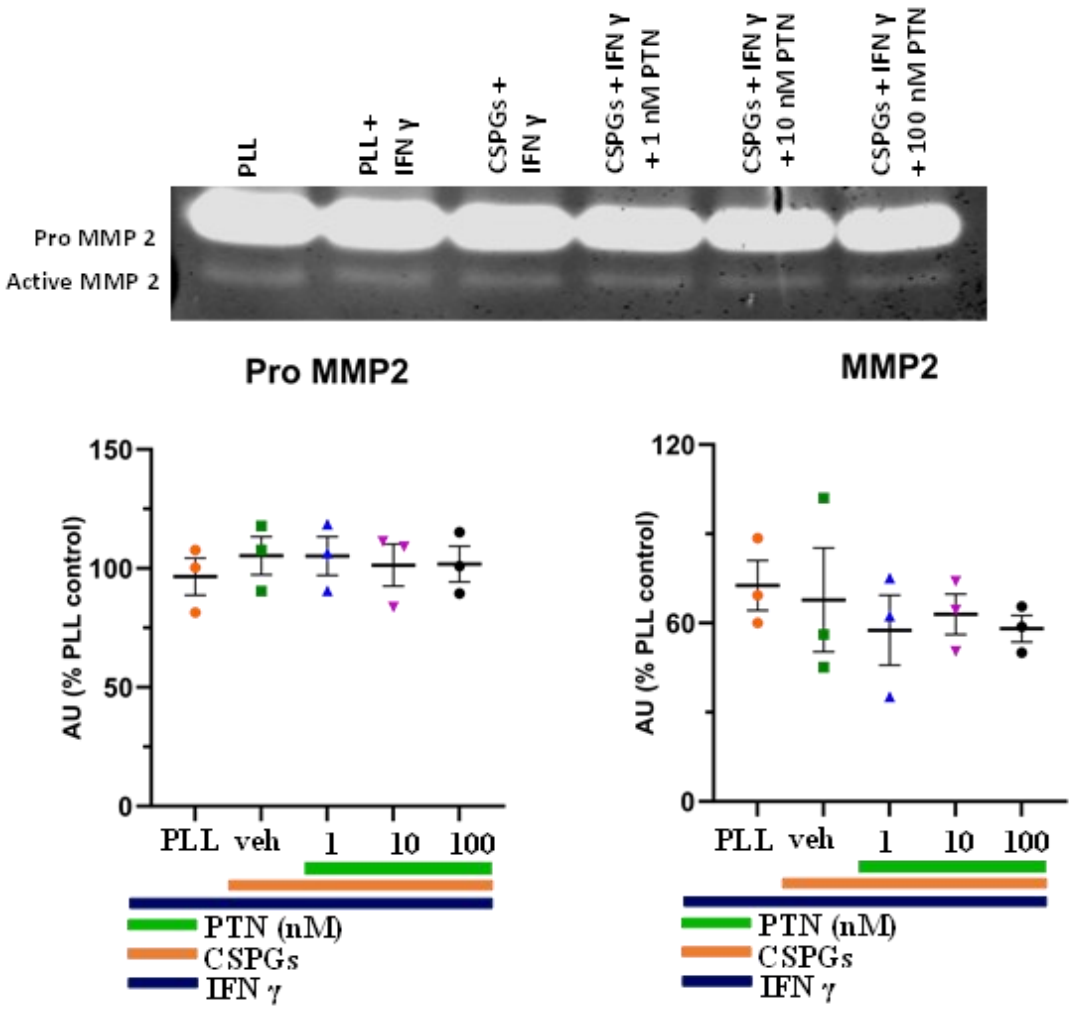


Figure 4.8: Effect of PTN treatment on the release MMP 9 and MMP 2 release from IFN γ stimulated mixed glia cultures.

Activity of MMP 9 and MMP 2 were detected using gel zymography from conditioned media from IFN γ stimulated mixed glia cultures treated with or without PTN. The intensities of the white band for (A) pro-MMP 9, active MMP 9, and (D) pro-MMP 2, active MMP 2 were calculated using Image J software. The intensities of PLL control were considered 100% for pro MMP 9, active MMP 9, Pro MMP 2, and active MMP 2. PTN did not show any difference in the levels of (B) pro-MMP 9 ($F_{(4, 10)} = 46.41$ $p < 0.0001$), (C) active MMP 9 ($F_{(4, 10)} = 1.868$ $p = 0.1928$), (E)

pro-MMP 2 ($F_{(4, 10)} = 0.1984$ $p = 0.9335$) and (F) active MMP 2 ($F_{(4, 10)} = 0.3584$ $p = 0.8326$) levels. (B – C, E – F) Error bars represent the standard error of the mean (SEM), and symbols **** represent $p < 0.001$, respectively. All data are based on 3 independent experiments with a minimum of 3 technical repeats.

Chapter 5 : Summary, conclusions, and future work

Recovery of neuronal connections after a stroke is essential for restoration of lost function. However, reparative processes are incomplete in the central nervous system (CNS), and most patients are left with permanent disability [305–307]. For improved recovery following brain injury due to stroke, several processes need to be initiated and augmented:

(1) Neurogenesis and neuritogenesis – generation of new neurons and regeneration of connectivity by new or repaired neurites.
(2) Axonal growth and myelination – myelinating the axons of existing and newly differentiated neurons.
(3) Reducing neuroinflammation during later phases of recovery
(4) Angiogenesis – formation of new blood vessels to provide support to regenerating tissue.

Neurogenesis and neuritogenesis

Neurogenesis is the process through which new neurons are generated in brain. It primarily occurs in specific regions of the healthy brain, in particular the subventricular zone (SVZ) and subgranular zone (SGZ) [308,309]. In the uninjured brain, neurogenesis is thought to be involved in learning and memory[310].

Following stroke, neurogenesis may play an important role in shaping the recovery after stroke. Neurons are cell most vulnerable to reductions in the supply of blood and oxygen to the brain and can die within 5 minutes of ischemic onset, disrupting networks essential to behaviour [311]. Activated microglia and reactive astrocytes (RAs) express stromal-derived factor 1α (SDF-1α) which acts as a chemokine for neuroblasts expressing C-X-C chemokine receptor type 4 (CXCR-4), thus attracting neuroblasts towards the damaged area [312]. Moreover, monocyte chemoattractant factor 1 (MCP-1) released from activated microglia and RAs also attract C-C chemokine receptor type 2 (CCR2) expressing neuroblasts to the damaged area [312] · During the initial weeks after a

stroke, RAs extend their processes, connecting the damaged ischemic tissue to SVZ, immature undeveloped neurons move along a grid-like network of astrocytes and migrate to damaged area [313]. Neurogenesis may then contribute in repair and regeneration of the damaged networks by forming new neurons and integrating newly generated neurons replacing dead neurons [312].

Neuritogenesis is the process of extending the growth of neurites from existing neurons and newly generated neurons after stroke. Extending neurites, including dendrites and axons, enables neurons to form synaptic connections with other neurons to restores communication [311]. Depending on the region affected, there is functional impairment that exhibits some degree of recovery with time based on the restoration of lost connectivity [306]. Newly generated neurons may integrate into existing circuitry essential for functions lost to stroke [17].

Axonal growth and myelination

In addition to newborn neurons, surviving neurons can form new neuronal connections by enhanced neurite growth including axonal sprouting [314]. These new axons often require myelination for optimal function. Myelination is a process of wrapping neurons with myelin sheath, which is produced by oligodendrocytes in central nervous system (CNS) [315]. Myelination increases the speed and efficiency of conducting nerve impulses [315]. Oligodendrocytes are generated by differentiation of Oligodendrocytes precursor cells (OPCs) [88]. After stroke, damaged oligodendrocytes cannot myelinate the neurons and thus new oligodendrocytes are required to myelinate the existing and newly generated neurons [316]. This remyelination process is essential for establishing proper nerve impulse conduction which is essential for the restoration of function. As noted, after stroke neurogenesis leads to the generation of new neurons and the extension of neurite growth from existing neurons to form new connections [309]. In the stroke recovery process,

myelination of these projections is essential to restore more normal communication of nerve impulses necessary to restore impaired motor function and cognitive processes [17].

Neuroinflammation

Neuroinflammation is the brain's immune response to damage or injury. The drivers of neuroinflammation includes the CNS resident immune cells, microglia, and other glial cells including astrocytes and oligodendrocytes that contribute to neuroinflammation directly and modulate the activity of microglia by releasing various growth factors and cytokines [317]. In the peripheral nervous system (PNS), the resident Schwann cells also produce pro inflammatory cytokines which further activates resident macrophages and fibroblasts [318] . There are two distinct phases of neuroinflammation: Acute neuroinflammation corresponds to an immediate phase after stroke where activated glial cells release various neuroprotective growth factors (BDNF, NGF, GDNF), anti inflammatory cytokines (IL 10, TGF β), pro inflammatory cytokines (IL 6, TNF, IL 1β and MCP 1), and extra cellular matrix components (chondroitin sulfate proteoglycans (CSPGs), fibronectin, collagens and Tenascin – C) [319–322]. Initial acute neuroinflammation is essential for clearing up the cellular debris and promoting repair after damage [321]. However, prolongation of this inflammation can lead to secondary damage [320]. Chronic inflammation can persist for months after stroke [72]. Sustained inflammation could be due to the continued activated states of microglia, which release pro-inflammatory cytokines that modulate the recovery process [72,321]. During the early stage of injury, activated microglia become localized in the injured area, but over time microglia can migrate into undamaged parts of the brain and contribute to secondary injury. In patients with stroke in the corticospinal tract, activated microglia were found in the thalamus and CST near brain stem two weeks post injury [323]. In the cortical stroke model, secondary thalamic injury is induced by microglia mediated neuroinflammation [324]**.** Prolonged activation of microglial

cells could potentially be associated with the loss of myelin even as long as one year following the injury, and in animal models, microglial depletion enhances white matter myelination after stroke [324].

Angiogenesis

Angiogenesis refers to the sprouting of new blood vessels from pre existing vessels. After stroke, angiogenesis is essential to restore blood flow to regions adjacent the damaged part of the brain, serving a role in cell survival and tissue repair [325]. Newly differentiated neurons and exiting neurons are vulnerable to supply of nutrients, and angiogenesis therefore plays an important role in protecting the viability of surviving and newborn neurons [326].

Identifying a single protein that can modulate neurogenesis/neuriteogenesis, neuroinflammation, axonal sprouting and myelination, and angiogenesis could be of great therapeutic value. Using *in vitro* models, this thesis investigated the potential effect of protein pleiotrophin (PTN) on neurons, OPCs, microglia, and astrocytes to modulate neurogenesis/neuriteogenesis, neuroinflammation, and myelination process.

PTN restores the growth of neurons in the presence of CSPGs

Chapter 2 tested the effect of PTN to overcome the inhibitory activity of CSPGs and enhance neurite outgrowth in neurons growing in an inhibitory environment. Activated glial cells are important in forming glial scars around a lesion to restrict the spread of damage to healthy parts of brain [207,327]. CSPGs are one of the main components of glial scar but are also known to inhibit the growth of neurons [207]. Eliminating CSPGs early after CNS injury leads to disrupted glial scar, expansion of core, and worse outcome 213, 328213, 328. However, eliminating CSPGs later in the recovery process is associated with enhanced neuroplasticity and improved functional recovery

[166]. Our data show that the growth of neurons in vitro is restricted in the presence of CSPGs, and that growth was enhanced with PTN treatment, with a peak response at a concentration of 15 ng mL^{-1} (Figure 2.1 K). PTN treatment also increased the complexity of outgrowth (Figure 2.1 L) and the number of neurons with neurite outgrowth (Figure 2.1 M). Moreover, the data show that the growth-promoting effect of PTN was blocked when the neurons were treated with alectinib (ALK antagonist) (Figure 2.2 P). Alectinib also reduced neurite growth complexity in PTN's presence (Figure 2.2 Q). This is the first study to demonstrate the involvement of ALK receptor in neuronal PTN signalling in the presence of CSPGs.

The findings of this study provide substantial evidence in favour of the hypothesis that PTN promotes neurite growth in the presence of CSPGs by signaling through the ALK receptor. The data show that different concentrations of PTN induce the growth of neurons *in vitro* in the presence of CSPGs, with an optimal effect at 15 ng mL^{-1} PTN. Considering *in vivo* conditions, PTN treatment could therefore have the potential to induce neuronal growth in the penumbra region where CSPGs are enriched after stroke. However, translation to the in vivo situation would require careful investigation of delivery methods, half life, and optimal dose. Alternatively, identifying key receptors and finding ligands for those receptors could benefit stroke recovery.

This study therefore investigated the involvement of the ALK receptor in PTN signalling using alectinib (ALK inhibitor) and SC79 (an Akt activator to mimic signaling downstream of ALK). As noted, alectinib data suggests that the ALK receptor is important to neuronal sprouting in a CSPG rich environment. To further confirm these findings, primary neuronal cultures can be further treated with protein-tyrosine phosphatase beta/zeta (RPTPβ/ ζ) antagonist [329] to silence the signalling of RPTPβ/ ζ. This information will help us to understand whether PTN signalling occurs through direct binding to ALK or by inhibiting the phosphatase activity of RPTPβ/ ζ.

PTN induces differentiation of OPCs to oligodendrocytes in the presence of CSPGs

Chapter 3 investigated the effect of PTN on the differentiation of OPCs to oligodendrocytes in the presence of CSPGs. CSPGs inhibit the growth of OPCs and differentiation to oligodendrocytes [54,133]. The data show that PTN induces the differentiation of OPCs to oligodendrocytes (Figure 3.2 A – D). SHOLL analysis data show that PTN also induced the complex process outgrowth from oligodendrocytes (Figure 3.3 B – D).

That PTN has the potential to overcome the inhibition of CSPGs and induce the differentiation of OPCs to oligodendrocytes is a significant development in studies of post-stroke plasticity and recovery. The data also indicate that PTN potentiates the process outgrowth from OPCs and oligodendrocytes, potentially allowing for greater myelination. Higher concentrations of PTN resulted in more OPCs compared to lower concentrations. This effect could be attributed to PTN's ability to block the binding of CSPGs to CSPG receptors or its capacity to induce OPC proliferation. To identify PTN's proliferative effect on OPCs, a proliferation assay can be conducted to determine the proliferative OPC population. Another approach to identify the impact of PTN on blocking CSPG binding to CSPG receptors involves using CSPG signaling inhibitors in the absence of PTN and quantifying the total number of OPCs. This data would help us better understand PTN's role in OPC proliferation.

PTN modulates the expression of cytokines and MMPs from microglia in the presence of CSPGs

Chapter 3 further investigated the effect of PTN on the expression of pro-inflammatory cytokines from microglia in the presence of CSPGs. As mentioned in previous literature, the data

show that CSPGs reduce the expression of pro-inflammatory cytokines, upon treatment with PTN, the expression of pro-inflammatory cytokines is further reduced (Figure 3.4 D – H). PTN also modulates the expression of the pro-inflammatory cytokines from microglia in the presence of inflammatory stimulus (IFN γ). The data from IFN γ stimulated condition show that CSPGs by themselves reduces the expression of pro-inflammatory cytokines, and the expression of pro inflammatory cytokines was increased when treated with PTN at higher concentration (10 nM and 100 nM PTN) which is essential during initial stage of injury (Figure 3.5 A – E). Along with the reduced expression of pro-inflammatory cytokines, CSPGs and PTN modulate the expression of pro MMPs and active MMPs from microglia. The data show that in the presence of CSPGs, PTN reduces the level of pro MMP 9 while increasing the level of MMP 9 (Figure 3.7 B – C). This higher level of MMP 9 could be due to increased expression of factors like MMP 13 and MMP 2, which converts the pro MMP 9 to MMP 9 [170]. Although with PTN treatment, we did not find any change in the levels of pro MMP 2, there was an increase in the level of MMP 2 (Figure 3.7 E – F). In IFN γ stimulated microglia, PTN treatment did not alter the expression of pro MMP 9, MMP 9, pro MMP 2 and MMP 2.

In summary, the data from this study suggests that PTN acts as has a dual effect on microglia in the presence of CSPGs. Under non inflammatory stimulus PTN reduces the expression of pro-inflammatory cytokines thus reducing inflammation. Moreover, PTN further induces the expression of MMP 9 and MMP 2, leading to cleavage of CSPGs. Under the inflammatory stimulus of IFN γ in the presence of CSPGs, PTN treatment induces the expression of pro-inflammatory cytokines, which is essential during early stage of injury Thus, the effects of PTN would likely be modulated by time after stroke and local inflammation.

In this study, the primary focus was to determine the effect of PTN on the release of pro-inflammatory cytokines. However, activated microglia are also known to release various anti-inflammatory cytokines (IL 10, TGF β, and IL 4) and growth factors (BDNF and NGF) [321], which should be assessed to gain a more comprehensive understanding of the effect of PTN on microglia in the presence of CSPGs. The data generated from this study indicates that PTN may serve as a potential therapeutic protein capable of reducing pro-inflammatory stimuli, but further study is required.

PTN modulates the expression of pro inflammatory cytokines from astrocytes and mixed glia

Chapter 4 investigated the effect of PTN on the release of pro inflammatory cytokines from astrocytes in the presence of CSPGs. Contrary to the effect of PTN on microglia in the presence of CSPGs, PTN induced the release of pro inflammatory cytokines (Figure 4.3 A – C) and reduced the levels of MMP 9 (Figure 4.4 C). We further explored the effect of PTN on mixed glia in the presence of CSPGs. The data show that with PTN, the expression of pro inflammatory cytokines is reduced at higher concentrations (10 nM and 100 nM PTN compared to 1 nM PTN) (Figure 4.5 A- C). Although there was a decrease in the amount of pro MMP 9 releases with PTN treatment, no difference was observed in the MMP 9 levels (Figure 4.7 B – C) suggesting that the increase in pro MMP 9 did not lead to greater conversion to active MMP 9. In inflammatory stimulus conditions, there was no significant difference in the expression of pro-inflammatory cytokines with PTN treatment, though there was a trend of increased expression of pro inflammatory cytokines (TNF, IL 6, MCP – 1 and IL 12p70) with higher concentration (Figure 4.6 A- D).

These studies highlight the complexities of studying inflammation in vitro, and the importance of cell-cell interactions in modulating inflammation. Overall, the data generated in this

study indicates that in the presence of CSPGs, PTN creates an inflammatory environment by inducing the release of pro-inflammatory cytokines from astrocytes. Additionally, PTN reduces the release of MMP 9 from astrocytes, resulting in a decrease in the digestion of CSPGs. Consequently, an inflammatory condition with reduced MMP expression is not a favorable environment for recovery. In a mixed glia environment, the PTN treatment at higher concentrations (10 nM and 100 nM) reduced the expression of pro-inflammatory cytokines compared to 1 nM PTN treatment (Figure 4.5 A - D). In Chapter 3, we observed that in the presence of CSPGs, PTN reduces the release of pro-inflammatory cytokines by microglia. In contrast, under the influence of CSPGs, PTN treatment triggers the release of pro-inflammatory cytokines by astrocytes. Therefore, in a mixed glial environment consisting of both microglia and astrocytes, the response to PTN treatment resembles the microglial response, suggesting that microglia can potentially modulate the effects of PTN on astrocytes.

PTN has been shown to protect hippocampal neurons against glutamate-induced toxicity [330], and further studies are required to understand the effect of PTN and CSPGs on glutamate uptake and release from astrocytes. While the data generated from this study demonstrates PTN's ability to modulate the response of microglia, reducing the release of pro-inflammatory cytokines and inducing the release of pro-inflammatory cytokines from astrocytes, additional investigations are needed. To explore the reduced effects of PTN on the release of pro-inflammatory cytokines in mixed glia conditions compared to microglia, PTN receptors and key signaling cascades in different glial cell types must be investigated. Whether PTN induces different responses in mixed versus isolated glial cell experiments is due to competing interactions on individual glial cells or modulation of one glial cells activity by other activated glia remains to be determined. Further, the potential of PTN to enhance differentiation of OPCs to oligodendrocytes in mixed glia

environment should be investigated to further demonstrate the role of PTN on differentiation in an environment more closely approximating the CNS.

In summary, our data shows that PTN can interact with neurons, microglia, OPCs, and astrocytes, modulating their activity in the presence of CSPGs. These studies demonstrate PTN as a potential candidate that can induce the processes of neuritogenesis, myelination, and the reduction of neuroinflammation, all of which are essential for effective regeneration after brain injuries such as stroke. Extension of these findings into an *in vivo* stroke model would be an important future step. We predict that treatment with PTN would lead to the differentiation of OPCs into mature oligodendrocytes in the CNS. These differentiated oligodendrocytes may then facilitate the myelination of developing neurons, ultimately contributing to recovery after a stroke. This could be tested in a focal model of stroke, such as photothrombosis, with assessment of sensorimotor recovery and myelination after stroke. *In vivo* imaging of myelin via SCoRE imaging could also reveal dynamics of myelination with or without PTN [331]. While motor skills or cognitive capabilities can both be impaired by stroke and exhibit some degree of spontaneous recovery, rehabilitative training is important to augment spontaneous recovery by linking neuroplasticity to restore connectivity with function [17,305]. Thus, it would be important to include rehabilitation in *in vivo* studies to determine benefits of enhanced plasticity.

Immediately after injury during the acute inflammatory state, treatment with PTN may enhance the inflammatory activity of microglia. Microglial activity is reduced by CSPGs which may be elevated adjacent the stroke, and PTN may thereby amplify microglial clearance cellular debris. During later stages of injury and recovery, where chronic inflammation persists, PTN treatment may reduce the release of pro-inflammatory cytokines from microglia, consequently reducing inflammation. Furthermore, PTN treatment can also induce the release of MMPs, which

can cleave CSPGs in the glial scar, creating an environment conducive to neuronal growth and OPC differentiation into oligodendrocytes, ultimately improving recovery after a stroke.

Thus, the data generated from this thesis indicates that the inhibitory activity of CSPGs can be overcome by PTN treatment, modulating the activity in neurons, OPCs, and microglia to create an environment conducive to regeneration and recovery. Further in vivo research is necessary before considering PTN as a potential therapeutic target to enhance recovery after a stroke or brain injury.

Effectors	Neurons	OPCs	Microglia	Astrocytes	Mixed glia
CSPGs and PTN	promotes growth	promotes differentiation	↓↓ pro inflammatory cytokines ↑↑ MMP9 and MMP 2	↑↑ Pro inflammatory cytokines ↓↓ MMP9 and MMP 2	↓ pro inflammatory cytokines ↑ MMP9 and MMP 2

Figure 5.1: Summary of effect of PTN on neurons, OPC, astrocytes and microglia

All these studies together indicates that PTN can overcome the CSPGs mediated inhibition on neuronal outgrowth, promote differentiation of OPCs into oligodendrocytes, reduce the pro inflammatory response from microglia and mixed glia, and induce the release of MMPs facilitating cleavage of CSPGs.

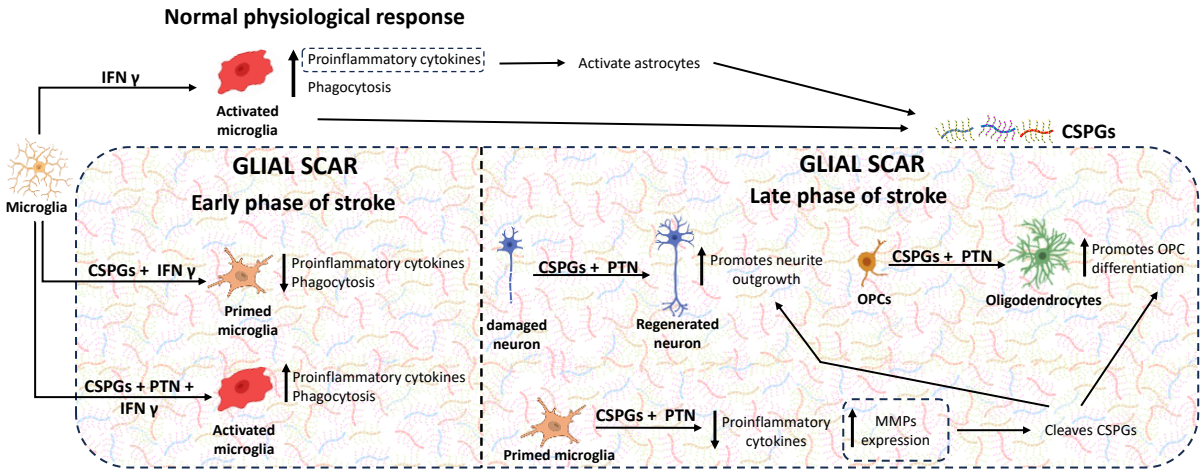


Figure 5.2: PTN can have favourable outcome during early and late phase of stroke

In the context of stroke, the pro-inflammatory stimulus elicited during the injury triggers the activation of astrocytes and microglia, leading to an increased release of CSPGs contributing in the formation of a glial scar around the lesion site. The early phase of stroke is characterized by a pro-inflammatory environment marked by the presence of IFN γ. During this stage, CSPGs exhibit a dampening effect on the pro-inflammatory activity of microglia, reducing their phagocytic activity. However, it is crucial to note that in the early phase of stroke, microglia tend to be activated with heightened phagocytotic activity. Treatment with PTN can effectively restore the pro-inflammatory and phagocytotic activity of microglia, facilitating the clearance of cellular debris. As the stroke progresses into the late phase, PTN demonstrates the ability to overcome the inhibitory influence of CSPGs within the glial scar. During the late phase of stroke, PTN plays a pivotal role in not only mitigating the inhibitory effects of CSPGs but also promoting the regeneration of damaged neurons. Additionally, PTN enhances the differentiation of OPCs into oligodendrocytes. Chronic inflammation in the late phase of stroke poses a risk of continued neuronal damage and treatment with PTN can be beneficial by reducing the pro-inflammatory activity of microglia. Furthermore, PTN can induce the release of MMPs, which can cleave CSPGs within the glial scar. This process promotes neuronal regeneration and facilitates the differentiation

of OPCs into oligodendrocytes. Overall, PTN emerges as a promising therapeutic agent capable of addressing multiple facets of the complex inflammatory and regenerative processes associated with stroke.

References

1. Murphy, T. H. & Corbett, D. Plasticity during stroke recovery: From synapse to behaviour. *Nat Rev Neurosci* **10**, 861–872 (2009).

2. Katan, M. & Luft, A. Global Burden of Stroke. *Semin Neurol* **38**, 208–211 (2018).

3. Tapuwa, D., Musuka, M., Wilton, S. & Traboulsi, M. Diagnosis and management of acute ischemic stroke: speed is critical. *Cmaj* **187**, 887–893 (2015).

4. Carmichael, S. T., Kathirvelu, B., Schweppe, C. A. & Nie, E. H. Molecular, cellular and functional events in axonal sprouting after stroke. *Exp Neurol* **287**, 384–394 (2017).

5. Carey, L. M., Matyas, T. A. & Oke, L. E. Sensory loss in stroke patients: Effective training of tactile and proprioceptive discrimination. *Arch Phys Med Rehabil* **74**, 602–611 (1993).

6. Lo, E. H. A new penumbra: Transitioning from injury into repair after stroke. *Nat Med* **14**, 497–500 (2008).

7. Huang, L. *et al.* Glial scar formation occurs in the human brain after ischemic stroke. *Int J Med Sci* **11**, 344–348 (2014).

8. Stankowski, J. N. & Gupta, R. Therapeutic targets for neuroprotection in acute ischemic stroke: lost in translation? *Antioxid Redox Signal* **14**, 1841–51 (2011).

9. Ma, H. *et al.* Thrombolysis Guided by Perfusion Imaging up to 9 Hours after Onset of Stroke. *N Engl J Med* **380**, 1795–1803 (2019).

10. Gravanis, I. & Tsirka, S. E. Tissue-type plasminogen activator as a therapeutic target in stroke. *Expert Opin Ther Targets* **12**, 159–170 (2008).

11. Psychogios, M. N. *et al.* Alberta stroke program early CT scale evaluation of multimodal computed tomography in predicting clinical outcomes of stroke patients treated with aspiration thrombectomy. *Stroke* **44**, 2188–2193 (2013).

12. Nie, X. *et al.* Futile Recanalization after Endovascular Therapy in Acute Ischemic Stroke. *BioMed Research International* vol. 2018 Preprint at https://doi.org/10.1155/2018/5879548 (2018).

13. Hussein, H. M. *et al.* Occurrence and predictors of futile recanalization following endovascular treatment among patients with acute ischemic stroke: A multicenter study. *American Journal of Neuroradiology* **31**, 454–458 (2010).

14. Citri, A. & Malenka, R. C. Synaptic plasticity: Multiple forms, functions, and mechanisms. *Neuropsychopharmacology* **33**, 18–41 (2008).

15. Cramer, S. C. & Nudo, R. J. Brain repair after stroke. *Brain Repair After Stroke* 1–296 (2010) doi:10.1017/CBO9780511777547.

16. Angels Font, M., Arboix, A. & Krupinski, J. Angiogenesis, Neurogenesis and Neuroplasticity in Ischemic Stroke. *Curr Cardiol Rev* **6**, 238–244 (2010).

17. Winship, I. R. & Murphy, T. H. Remapping the somatosensory cortex after stroke: insight from imaging the synapse to network. *Neuroscientist* **15**, 507–524 (2009).

18. Merson, T. D. & Bourne, J. A. Endogenous neurogenesis following ischaemic brain injury: Insights for therapeutic strategies. *International Journal of Biochemistry and Cell Biology* vol. 56 4–19 Preprint at https://doi.org/10.1016/j.biocel.2014.08.003 (2014).

19. Zhang, R. L., Zhang, Z. G., Zhang, L. & Chopp, M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience* **105**, 33–41 (2001).

20. Guerra-Crespo, M. *et al.* Transforming growth factor-alpha induces neurogenesis and behavioral improvement in a chronic stroke model. *Neuroscience* **160**, 470–483 (2009).

21. Zhang, Z., Zhang, N. & Ding, S. Glial cell line-derived neurotrophic factor in brain repair after focal ischemic stroke. *Neural Regen Res* **17**, 1735 (2022).

22. Mu, J. D. *et al.* The factors affecting neurogenesis after stroke and the role of acupuncture. *Front Neurol* **14**, (2023).

23. Huebner, E. A. & Strittmatter, S. M. Axon Regeneration in the Peripheral and Central Nervous Systems. *Results Probl Cell Differ* **48**, 339 (2009).

24. Carmichael, S. T., Kathirvelu, B., Schweppe, C. A. & Nie, E. H. Molecular, Cellular and Functional Events in Axonal Sprouting after Stroke. *Exp Neurol* **287**, 384 (2017).

25. Murphy, T. H. & Corbett, D. Plasticity during stroke recovery: From synapse to behaviour. *Nature Reviews Neuroscience* vol. 10 861–872 Preprint at https://doi.org/10.1038/nrn2735 (2009).

26. Alia, C. *et al.* Neuroplastic Changes Following Brain Ischemia and their Contribution to Stroke Recovery: Novel Approaches in Neurorehabilitation. *Front Cell Neurosci* **11**, (2017).

27. Mukherjee, N. & Ghosh, S. Myelin Associated Inhibitory Proteins as a Therapeutic Target for Healing of CNS Injury. *ACS Chemical Neuroscience* vol. 11 1699–1700 Preprint at https://doi.org/10.1021/acschemneuro.0c00280 (2020).

28. Debellard, M.-E., Tang, S., Mukhopadhyay, G., Shen, Y.-J. & Filbin, M. T. *MCN Myelin-Associated Glycoprotein Inhibits Axonal Regeneration from a Variety of Neurons via Interaction with a Sialoglycoprotein*. *Molecular and Cellular Neuroscience* vol. 7 (1996).

29. Sainath R, Ketschek A, Grandi L, G. G. CSPGs Inhibit Axon Branching by Impairing Mitochondria- Dependent Regulation of Actin Dynamics and Axonal Translation. *Dev Neurobiol.* **176**, 139–148 (2017).

30. Hosseini, S. M. *et al.* Suppressing CSPG/LAR/PTPr Axis Facilitates Neuronal Replacement and Synaptogenesis by Human Neural Precursor Grafts and Improves Recovery after Spinal Cord Injury. *Journal of Neuroscience* **42**, 3096–3121 (2022).

31. Luo, F. *et al.* Inhibition of CSPG receptor PTPσ promotes migration of newly born neuroblasts, axonal sprouting, and recovery from stroke. *Cell Rep* **40**, (2022).

32. Hettiaratchi, M. H. *et al.* Local delivery of stabilized chondroitinase ABC degrades chondroitin sulfate proteoglycans in stroke-injured rat brains. *Journal of Controlled Release* **297**, 14–25 (2019).

33. Paveliev, M. *et al.* HB-GAM (pleiotrophin) reverses inhibition of neural regeneration by the CNS extracellular matrix. *Sci Rep* **6**, 1–17 (2016).

34. Carmichael, S. T. *et al.* Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. *Exp Neurol* **193**, 291–311 (2005).

35. Bonilla, I. E., Tanabe, K. & Strittmatter, S. M. Small Proline-Rich Repeat Protein 1A Is Expressed by Axotomized Neurons and Promotes Axonal Outgrowth. *The Journal of Neuroscience* **22**, 1303 (2002).

36. Xu, X. H. *et al.* MARCKS regulates membrane targeting of Rab10 vesicles to promote axon development. *Cell Res* **24**, 576–594 (2014).

37. Poulain, F. E. & Sobel, A. The "SCG10-LIke Protein" SCLIP is a novel regulator of axonal branching in hippocampal neurons, unlike SCG10. *Molecular and Cellular Neuroscience* **34**, 137–146 (2007).

38. Blackburn, D., Sargsyan, S., Monk, P. N. & Shaw, P. J. Astrocyte function and role in motor neuron disease: A future therapeutic target? *Glia* **57**, 1251–1264 (2009).

39. Anderson, M. F., Blomstrand, F., Blomstrand, C., Eriksson, P. S. & Nilsson, M. Astrocytes and stroke: Networking for survival? *Neurochem Res* **28**, 293–305 (2003).

40. Li, L. *et al.* The Specific Role of Reactive Astrocytes in Stroke. *Front Cell Neurosci* **16**, (2022).

41. Choi, J. K. *et al.* GM-CSF reduces expression of chondroitin sulfate proteoglycan (CSPG) core proteins in TGF-β-treated primary astrocytes. *BMB Rep* **47**, 679–684 (2014).

42. Gouix, E. *et al.* Oxygen glucose deprivation-induced astrocyte dysfunction provokes neuronal death through oxidative stress. *Pharmacol Res* **87**, 8–17 (2014).

43. Bains, J. S. & Shaw, C. A. Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Rev* **25**, 335–358 (1997).

44. Buffo, A., Rolando, C. & Ceruti, S. Astrocytes in the damaged brain: Molecular and cellular insights into their reactive response and healing potential. *Biochem Pharmacol* **79**, 77–89 (2010).

45. Patabendige, A., Singh, A., Jenkins, S., Sen, J. & Chen, R. Astrocyte activation in neurovascular damage and repair following ischaemic stroke. *Int J Mol Sci* **22**, (2021).

46. Luo, J. TGF-β as a Key Modulator of Astrocyte Reactivity: Disease Relevance and Therapeutic Implications. *Biomedicines* **10**, (2022).

47. Wiese, S., Karus, M. & Faissner, A. Astrocytes as a source for extracellular matrix molecules and cytokines. *Front Pharmacol* **3 JUN**, 1–13 (2012).

48. González-Castillo† Celia, Ortuño-Sahagún2†, D., Guzmán-Brambila3, C., Rojas-Mayorquín5* & Esperanza, M. P. and A. Pleiotrophin as a central nervous system neuromodulator, evidences from the hippocampus. *Front Cell Neurosci* **8**, 1–7 (2015).

49. Mi, R., Chen, W. & Höke, A. Pleiotrophin is a neurotrophic factor for spinal motor neurons. *Proc Natl Acad Sci U S A* **104**, 4664–4669 (2007).

50. Siebert, J. R., Conta Steencken, A. & Osterhout, D. J. Chondroitin Sulfate Proteoglycans in the Nervous System: Inhibitors to Repair. *Biomed Res Int* **2014**, (2014).

51. Pekny, M. *et al.* Abnormal Reaction to Central Nervous System Injury in Mice Lacking Glial Fibrillary Acidic Protein and Vimentin. *Journal of Cell Biology* **145**, 503–514 (1999).

52. Djerbal, L., Lortat-Jacob, H. & Kwok, J. Chondroitin sulfates and their binding molecules in the central nervous system. *Glycoconj J* **34**, 363–376 (2017).

53. DeWitt, D. A., Richey, P. L., Praprotnik, D., Silver, J. & Perry, G. Chondroitin sulfate proteoglycans are a common component of neuronal inclusions and astrocytic reaction in neurodegenerative diseases. *Brain Res* **656**, 205–209 (1994).

54. Pendleton, J. C. *et al.* Chondroitin sulfate proteoglycans inhibit oligodendrocyte myelination through PTPσ. *Exp Neurol* **247**, 113–121 (2013).

55. Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S. & Nabekura, J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *Journal of Neuroscience* **29**, 3974–3980 (2009).

56. Gadani, S. P., Walsh, J. T., Lukens, J. R., Kipnis, J. & Program, S. T. Dealing with Danger in the CNS: The Response of the Immune System to Injury. **87**, 47–62 (2016).

57. Honda, S. *et al.* Extracellular ATP or ADP Induce Chemotaxis of Cultured Microglia through G i/o -Coupled P2Y Receptors . *The Journal of Neuroscience* **21**, 1975–1982 (2001).

58. Färber, K. & Kettenmann, H. Purinergic signaling and microglia. *Pflugers Arch* **452**, 615–621 (2006).

59. Rodrigues, R. J., Tomé, A. R. & Cunha, R. A. ATP as a multi-target danger signal in the brain. *Front Neurosci* **9**, 1–11 (2015).

60. Y. Ueda, MD; T.P. Obrenovitch, PhD; S.Y. Lok, Bs. & G.S. Sarna, PhD; and L. Symon, TD, F. Changes in extracellular glutamate concentration produced in the rat striatum by repeated ischemia. *Stroke* **24**, 912–913 (1993).

61. Mead, E. L. *et al.* Microglial neurotransmitter receptors trigger superoxide production in microglia; consequences for microglial-neuronal interactions. *J Neurochem* **121**, 287–301 (2012).

62. Helmut, K., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol Rev* **91**, 461–553 (2011).

63. Ransohoff, R. M. A polarizing question: Do M1 and M2 microglia exist. *Nat Neurosci* **19**, 987–991 (2016).

64. Yenari, M. A., Kauppinen, T. M. & Swanson, R. A. Microglial Activation in Stroke: Therapeutic Targets. *Neurotherapeutics* **7**, 378–391 (2010).

65. Li-Jin Chew, Asako Takanohashi, and M. B. MICROGLIA AND INFLAMMATION:IMPACT ON DEVELOPMENTAL BRAIN INJURIES. **287**, 280–287 (2006).

66. Jiang, C. T., Wu, W. F., Deng, Y. H. & Ge, J. W. Modulators of microglia activation and polarization in ischemic stroke (Review). *Mol Med Rep* **21**, 2006–2018 (2020).

67. Rawlinson, C., Jenkins, S., Thei, L., Dallas, M. L. & Chen, R. Post-ischaemic immunological response in the brain: Targeting microglia in ischaemic stroke therapy. *Brain Sci* **10**, (2020).

68. Taylor, R. A. & Sansing, L. H. Microglial responses after ischemic stroke and intracerebral hemorrhage. *Clin Dev Immunol* **2013**, (2013).

69. Jian, Z. *et al.* The Involvement and Therapy Target of Immune Cells After Ischemic Stroke. *Front Immunol* **10**, 1–15 (2019).

70. Patel, A. R., Ritzel, R., McCullough, L. D. & Liu, F. Microglia and ischemic stroke: A double-edged sword. *Int J Physiol Pathophysiol Pharmacol* **5**, 73–90 (2013).

71. Ransohoff, R. M. A polarizing question: Do M1 and M2 microglia exist. *Nat Neurosci* **19**, 987–991 (2016).

72. Anrather, J. & Iadecola, C. Inflammation and Stroke: An Overview. *Neurotherapeutics* **13**, 661–670 (2016).

73. Rogers, J. A. *et al.* An inhibitor of chondroitin sulfate proteoglycan synthesis promotes central nervous system remyelination. *Nat Commun* **7**, 1–12 (2016).

74. Hughes, E. G., Kang, S. H., Fukaya, M. & Bergles, D. E. Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat Neurosci* **16**, 668–676 (2013).

75. Bergles, D. E. & Richardson, W. D. Oligodendrocyte development and plasticity. *Cold Spring Harb Perspect Biol* **8**, 1–28 (2016).

76. Adams, K. L., Dahl, K. D., Gallo, V. & Macklin, W. B. Intrinsic and extrinsic regulators of oligodendrocyte progenitor proliferation and differentiation. *Semin Cell Dev Biol* **116**, 16–24 (2021).

77. Buchanan, J. A. *et al.* Oligodendrocyte precursor cells ingest axons in the mouse neocortex. *Proc Natl Acad Sci U S A* **119**, e2202580119 (2022).

78. Frederick, T. J., Min, J., Altieri, S. C., Mitchell, N. E. & Wood, T. L. Synergistic induction of cyclin D1 in oligodendrocyte progenitor cells by IGF-I and FGF-2 requires differential stimulation of multiple signaling pathways. *Glia* **55**, 1011–1022 (2007).

79. Morell, P. & Toews, A. D. In Vivo Metabolism of Oligodendroglial Lipids. *Oligodendroglia* 47–86 (1984) doi:10.1007/978-1-4757-6066-8_2.

80. Waly, B. El, Macchi, M., Cayre, M. & Durbec, P. Oligodendrogenesis in the normal and pathological central nervous system. *Front Neurosci* **8**, 1–22 (2014).

81. Chapman, T. W., Olveda, G. E., Pereira, E. & Hill, R. A. Age and axon-specific forms of cortical remyelination by divergent populations of NG2-glia. *bioRxiv* 1–24 (2020) doi:10.1101/2020.12.09.414755.

82. Baydyuk, M., Morrison, V. E., Gross, P. S. & Huang, J. K. Extrinsic Factors Driving Oligodendrocyte Lineage Cell Progression in CNS Development and Injury. *Neurochemical Research 2020 45:3* **45**, 630–642 (2020).

83. Zhang, R., Chopp, M. & Zhang, Z. G. Oligodendrogenesis after cerebral ischemia. *Front Cell Neurosci* **7**, (2013).

84. Zhang, R., Chopp, M. & Zhang, Z. G. Oligodendrogenesis after cerebral ischemia. *Front Cell Neurosci* **7**, (2013).

85. Miron, V. E. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nature Neuroscience 2013 16:9* **16**, 1211–1218 (2013).

86. Jablonska, B. *et al.* Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination. *Nat Neurosci* **13**, 541–550 (2010).

87. Dittmann, N. L., Torabi, P., Watson, A. E. S., Yuzwa, S. A. & Voronova, A. Culture Protocol and Transcriptomic Analysis of Murine SVZ NPCs and OPCs. *Stem Cell Rev Rep* **19**, 983–1000 (2023).

88. Dewar, D., Underhill, S. M. & Goldberg, M. P. Oligodendrocytes and ischemic brain injury. *Journal of Cerebral Blood Flow and Metabolism* **23**, 263–274 (2003).

89. Morell, P. & Toews, A. D. In Vivo Metabolism of Oligodendroglial Lipids. *Oligodendroglia* 47–86 (1984) doi:10.1007/978-1-4757-6066-8_2.

90. Fern, R. & Möller, T. Rapid Ischemic Cell Death in Immature Oligodendrocytes: A Fatal Glutamate Release Feedback Loop. *Journal of Neuroscience* **20**, 34–42 (2000).

91. Lin, W. *et al.* Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress. *Brain* **129**, 1306–1318 (2006).

92. Pu, A., Stephenson, E. L. & Yong, V. W. The extracellular matrix: Focus on oligodendrocyte biology and targeting CSPGs for remyelination therapies. *Glia* **66**, 1809–1825 (2018).

93. Shriver, Z., Capila, I., Venkataraman, G. & Sasisekharan, R. Heparin and Heparan Sulfate: Analyzing Structure and Microheterogeneity. *Handb Exp Pharmacol* **207**, 159 (2012).

94. Condomitti, G. & De Wit, J. Heparan Sulfate Proteoglycans as Emerging Players in Synaptic Specificity. *Front Mol Neurosci* **11**, (2018).

95. Ohtake, Y., Saito, A. & Li, S. Diverse functions of protein tyrosine phosphatase σ in the nervous and immune systems. *Experimental Neurology* vol. 302 196–204 Preprint at https://doi.org/10.1016/j.expneurol.2018.01.014 (2018).

96. Zhang, P. *et al.* Heparan Sulfate Organizes Neuronal Synapses through Neurexin Partnerships. *Cell* **174**, 1450-1464.e23 (2018).

97. Pu, A., Stephenson, E. L. & Yong, V. W. The extracellular matrix: Focus on oligodendrocyte biology and targeting CSPGs for remyelination therapies. *Glia* **66**, 1809–1825 (2018).

98. Properzi, F. *et al.* Heparan sulphate proteoglycans in glia and in the normal and injured CNS: expression of sulphotransferases and changes in sulphation. *Eur J Neurosci* **27**, 593–604 (2008).

99. Probstmeier, R. & Pesheva, P. Tenascin-C inhibits β 1 integrin-dependent cell adhesion and neurite outgrowth on fibronectin by a disialoganglioside-mediated signaling mechanism. *Glycobiology* **9**, 101–114 (1999).

100. Siddiqui, S., Horvat-Bröcker, A. & Faissner, A. The glia-derived extracellular matrix glycoprotein tenascin-C promotes embryonic and postnatal retina axon outgrowth via the alternatively spliced fibronectin type III domain TNfnD. *Neuron Glia Biol* **4**, 271–283 (2008).

101. Hanmin, C. *et al.* Pleiotropic Role of Tenascin-C in Central Nervous System Diseases: From Basic to Clinical Applications. *Front Neurol* **11**, 576230 (2020).

102. Tucić, M., Stamenković, V. & Andjus, P. The Extracellular Matrix Glycoprotein Tenascin C and Adult Neurogenesis. *Front Cell Dev Biol* **9**, (2021).

103. Tonge, D. A. *et al.* Fibronectin supports neurite outgrowth and axonal regeneration of adult brain neurons in vitro. *Brain Res* **1453**, 8 (2012).

104. Wang, J. T., Yin, L. & Chen, Z. Neuroprotective role of fibronectin in neuron-glial extrasynaptic transmission. *Neural Regen Res* **8**, 376 (2013).

105. McDonald, J. W. & Sadowsky, C. Spinal-cord injury. *Lancet* **359**, 417–425 (2002).

106. Ivins, J. K., Yurchenco, P. D. & Lander, A. D. Regulation of Neurite Outgrowth by Integrin Activation. *Journal of Neuroscience* **20**, 6551–6560 (2000).

107. Mohan, H. *et al.* Extracellular Matrix in Multiple Sclerosis Lesions: Fibrillar Collagens, Biglycan and Decorin are Upregulated and Associated with Infiltrating Immune Cells. *Brain Pathology* **20**, 966 (2010).

108. Soles, A. *et al.* Extracellular Matrix Regulation in Physiology and in Brain Disease. *Int J Mol Sci* **24**, (2023).

109. Toole, B. P. Hyaluronan in morphogenesis. *Semin Cell Dev Biol* **12**, 79–87 (2001).

110. Dityatev, A. & Schachner, M. Extracellular matrix molecules and synaptic plasticity. *Nat Rev Neurosci* **4**, 456–468 (2003).

111. Edgar, D., Timpl, R. & Thoenen, H. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO J* **3**, 1463–1468 (1984).

112. Cohen, J., Burne, J. F., McKinlay, C. & Winter, J. The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. *Dev Biol* **122**, 407–418 (1987).

113. Baron-Van Evercooren, A. *et al.* Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J Neurosci Res* **8**, 179–193 (1982).

114. Clemente, D., Ortega, M. C., Melero-Jerez, C. & de Castro, F. The effect of glia-glia interactions on oligodendrocyte precursor cell biology during development and in demyelinating diseases. *Front Cell Neurosci* **7**, 66678 (2013).

115. Buttery, P. C. & Ffrench-Constant, C. Laminin-2/Integrin Interactions Enhance Myelin Membrane Formation by Oligodendrocytes. *Molecular and Cellular Neuroscience* **14**, 199–212 (1999).

116. Siebert, J. R., Conta Steencken, A. & Osterhout, D. J. Chondroitin Sulfate Proteoglycans in the Nervous System: Inhibitors to Repair. *Biomed Res Int* **2014**, 1–15 (2014).

117. Fawcett, J. W. & Asher, R. A. The glial scar and central nervous system repair. *Brain Res Bull* **49**, 1–15 (1999).

118. Rauch, U. & Kappler, J. Chondroitin/Dermatan Sulfates in the Central Nervous System: Their Structures and Functions in Health and Disease. *Adv Pharmacol* **53**, 337–356 (2006).

119. Siebert, J. R., Conta Steencken, A. & Osterhout, D. J. Chondroitin Sulfate Proteoglycans in the Nervous System: Inhibitors to Repair. *Biomed Res Int* **2014**, (2014).

120. Properzi, F. *et al.* Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. *European Journal of Neuroscience* **21**, 378–390 (2005).

121. Lin, R., Rosahl, T. W., Whiting, P. J., Fawcett, J. W. & Kwok, J. C. F. 6-Sulphated chondroitins have a positive influence on axonal regeneration. *PLoS One* **6**, (2011).

122. Wang, H. *et al.* Chondroitin-4-sulfation negatively regulates axonal guidance and growth. *J Cell Sci* **121**, 3083–3091 (2008).

123. Pearson, C. S., Mencio, C. P., Barber, A. C., Martin, K. R. & Geller, H. M. Identification of a critical sulfation in chondroitin that inhibits axonal regeneration. *Elife* **7**, 1–21 (2018).

124. Jones, L. L., Sajed, D. & Tuszynski, M. H. Axonal Regeneration through Regions of Chondroitin Sulfate Proteoglycan Deposition after Spinal Cord Injury: A Balance of Permissiveness and Inhibition. *Journal of Neuroscience* **23**, 9276–9288 (2003).

125. Sofroniew, M. V. Multiple roles for astrocytes as effectors of cytokines and inflammatory mediators. *Neuroscientist* **20**, 160–172 (2014).

126. Jin, J. *et al.* Effect of chondroitin sulfate proteoglycans on neuronal cell adhesion, spreading and neurite growth in culture. *Neural Regen Res* **13**, 0 (2018).

127. Ohtake, Y. & Li, S. Receptors of Chondroitin Sulfate Proteoglycans and CNS Repair. *Austin J Neurol Disord Epilepsy. Austin J Neurol Disord Epilepsy* **2**, 1010–1 (2015).

128. Ohtake, Y. & Li, S. Receptors of Chondroitin Sulfate Proteoglycans and CNS Repair. *Austin J Neurol Disord Epilepsy. Austin J Neurol Disord Epilepsy* **2**, 1010–1 (2015).

129. Sainath R, Ketschek A, Grandi L, G. G. CSPGs Inhibit Axon Branching by Impairing Mitochondria- Dependent Regulation of Actin Dynamics and Axonal Translation. *Dev Neurobiol.* **176**, 139–148 (2017).

130. Blanquie, O. & Bradke, F. Cytoskeleton dynamics in axon regeneration. *Curr Opin Neurobiol* **51**, 60–69 (2018).

131. Snow, D. M., Atkinson, P. B., Hassinger, T. D., Letourneau, P. C. & Kater, S. B. Chondroitin Sulfate Proteoglycan Elevates Cytoplasmic Calcium in DRG Neurons. *Dev Biol* **166**, 87–100 (1994).

132. Sun, Y. *et al.* Chondroitin sulfate proteoglycans inhibit the migration and differentiation of oligodendrocyte precursor cells and its counteractive interaction with laminin. *Int J Mol Med* **40**, 1657–1668 (2017).

133. Sun, Y. *et al.* Chondroitin sulfate proteoglycans inhibit the migration and differentiation of oligodendrocyte precursor cells and its counteractive interaction with laminin. *Int J Mol Med* **40**, 1657–1668 (2017).

134. Dyck, S. *et al.* Perturbing chondroitin sulfate proteoglycan signaling through LAR and PTPσ receptors promotes a beneficial inflammatory response following spinal cord injury. *J Neuroinflammation* **15**, 1–26 (2018).

135. Rolls, A. *et al.* Two faces of chondroitin sulfate proteoglycan in spinal cord repair: A role in microglia/ macrophage activation. *PLoS Med* **5**, 1262–1277 (2008).

136. Stephenson, E. L. & Yong, V. W. Pro-inflammatory roles of chondroitin sulfate proteoglycans in disorders of the central nervous system. *Matrix Biology* **71–72**, 432–442 (2018).

137. Zhang, W., Sun, F., Niu, H., Wang, Q. & Duan, J. Mechanistic insights into cellular immunity of chondroitin sulfate A and its zwitterionic N-deacetylated derivatives. *Carbohydr Polym* **123**, 331–338 (2015).

138. Zheng, P. S. *et al.* PG-M/versican binds to P-selectin glycoprotein ligand-1 and mediates leukocyte aggregation. *J Cell Sci* **117**, 5887–5895 (2004).

139. Stephenson, E. L. & Yong, V. W. Pro-inflammatory roles of chondroitin sulfate proteoglycans in disorders of the central nervous system. *Matrix Biology* **71–72**, 432–442 (2018).

140. Bourdon, M. A., Krusius, T., Campbell, S., Schwartz, N. B. & Ruoslahti, E. Identification and synthesis of a recognition signal for the attachement of glycosaminoglycans to proteins. *Proc Natl Acad Sci U S A* **84**, 3194–3198 (1987).

141. Esko, J. D. & Zhangt, L. Influence of core protein sequence on glycosaminoglycan assembly. *Curr Opin Struct Biol* **6**, 663–670 (1996).

142. Nigro, J. *et al.* Regulation of heparan sulfate and chondroitin sulfate glycosaminoglycan biosynthesis by 4-fluoro-glucosamine in murine airway smooth muscle cells. *Journal of Biological Chemistry* **284**, 16832–16839 (2009).

143. Keough, M. B. *et al.* An inhibitor of chondroitin sulfate proteoglycan synthesis promotes central nervous system remyelination. *Nature Communications 2016 7:1* **7**, 1–12 (2016).

144. Sami, A., Selzer, M. E. & Li, S. Advances in the Signaling Pathways Downstream of Glial-Scar Axon Growth Inhibitors. *Front Cell Neurosci* **14**, 1–12 (2020).

145. Dill, J., Wang, H., Zhou, F. & Li, S. Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. *J Neurosci* **28**, 8914–8928 (2008).

146. Lang, B. T. *et al.* Modulation of the proteoglycan receptor PTPσ promotes recovery after spinal cord injury. *Nature* **518**, 404–408 (2015).

147. Luo, F. *et al.* Modulation of proteoglycan receptor PTPσ enhances MMP-2 activity to promote recovery from multiple sclerosis. *Nat Commun* **9**, (2018).

148. Tran, A. P., Sundar, S., Yu, M., Lang, B. T. & Silver, J. Modulation of Receptor Protein Tyrosine Phosphatase Sigma Increases Chondroitin Sulfate Proteoglycan Degradation through Cathepsin B Secretion to Enhance Axon Outgrowth. *The Journal of Neuroscience* **38**, 5399 (2018).

149. Popat, R. J. *et al.* Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* **416**, 636–640 (2002).

150. Barritt, A. W. *et al.* Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. *Journal of Neuroscience* **26**, 10856–10867 (2006).

151. Hill, J. J., Jin, K., Mao, X. O., Xie, L. & Greenberg, D. A. Intracerebral chondroitinase ABC and heparan sulfate proteoglycan glypican improve outcome from chronic stroke in rats. *Proc Natl Acad Sci U S A* **109**, 9155–9160 (2012).

152. Wiersma, A. M., Fouad, K. & Winship, I. R. Enhancing Spinal Plasticity Amplifies the Benefits of Rehabilitative Training and Improves Recovery from Stroke. *The Journal of Neuroscience* **37**, 10983–10997 (2017).

153. Gherardini, L., Gennaro, M. & Pizzorusso, T. Perilesional treatment with chondroitinase ABC and motor training promote functional recovery after stroke in rats. *Cerebral Cortex* **25**, 202–212 (2015).

154. Mingorance, A. *et al.* Regeneration of lesioned entorhino-hippocampal axons in vitro by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NgR signaling. *FASEB J* **20**, 491–493 (2006).

155. Karimi-Abdolrezaee, S., Schut, D., Wang, J. & Fehlings, M. G. Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury. *PLoS One* **7**, 37589 (2012).

156. Siebert, J. R., Stelzner, D. J. & Osterhout, D. J. Chondroitinase treatment following spinal contusion injury increases migration of oligodendrocyte progenitor cells. *Exp Neurol* **231**, 19–29 (2011).

157. Karimi-Abdolrezaee, S., Schut, D., Wang, J. & Fehlings, M. G. Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury. *PLoS One* **7**, 37589 (2012).

158. Pendleton, J. C. *et al.* Chondroitin sulfate proteoglycans inhibit oligodendrocyte myelination through PTPσ. *Exp Neurol* **247**, 113–121 (2013).

159. Bartus, K. *et al.* Large-scale chondroitin sulfate proteoglycan digestion with chondroitinase gene therapy leads to reduced pathology and modulates macrophage phenotype following spinal cord contusion injury. *Journal of Neuroscience* **34**, 4822–4836 (2014).

160. Didangelos, A., Iberl, M., Vinsland, E., Bartus, K. & Bradbury, E. J. Regulation of IL-10 by chondroitinase ABC promotes a distinct immune response following spinal cord injury. *Journal of Neuroscience* **34**, 16424–16432 (2014).

161. Rolls, A. *et al.* A sulfated disaccharide derived from chondroitin sulfate proteoglycan protects against inflammation-associated neurodegeneration. *FASEB J* **20**, 547–549 (2006).

162. Rolls, A. *et al.* A sulfated disaccharide derived from chondroitin sulfate proteoglycan protects against inflammation-associated neurodegeneration. *FASEB J* **20**, 547–549 (2006).

163. Lee, H., McKeon, R. J. & Bellamkonda, R. V. Sustained delivery of thermostabilized chABC enhances axonal sprouting and functional recovery after spinal cord injury. *Proc Natl Acad Sci U S A* **107**, 3340–3345 (2010).

164. Tester, N. J., Plaas, A. H. & Howland, D. R. Effect of body temperature on chondroitinase ABC's ability to cleave chondroitin sulfate glycosaminoglycans. *J Neurosci Res* **85**, 1110–1118 (2007).

165. Lee, H., McKeon, R. J. & Bellamkonda, R. V. Sustained delivery of thermostabilized chABC enhances axonal sprouting and functional recovery after spinal cord injury. *Proc Natl Acad Sci U S A* **107**, 3340–3345 (2010).

166. James, N. D. *et al.* Chondroitinase gene therapy improves upper limb function following cervical contusion injury. *Exp Neurol* **271**, 131–135 (2015).

167. Bartus, K. *et al.* Large-Scale Chondroitin Sulfate Proteoglycan Digestion with Chondroitinase Gene Therapy Leads to Reduced Pathology and Modulates Macrophage Phenotype following Spinal Cord Contusion Injury. *Journal of Neuroscience* **34**, 4822–4836 (2014).

168. Burnside, E. R. *et al.* Immune-evasive gene switch enables regulated delivery of chondroitinase after spinal cord injury. *Brain* **141**, 2362 (2018).

169. Könnecke, H. & Bechmann, I. The role of microglia and matrix metalloproteinases involvement in neuroinflammation and gliomas. *Clin Dev Immunol* **2013**, (2013).

170. L M Matrisian. The matrix-degrading metalloproteinases. *BioEssay* **153**, 545–548 (1962).

171. Yang, Y. & Rosenberg, G. A. Matrix metalloproteinases as therapeutic targets for stroke. *Brain Res* **1623**, 30 (2015).

172. Gorter, R. P. & Baron, W. Matrix metalloproteinases shape the oligodendrocyte (niche) during development and upon demyelination. *Neurosci Lett* **729**, 134980 (2020).

173. Larsen, P. H., DaSilva, A. G., Conant, K. & Yong, V. W. Myelin Formation during Development of the CNS Is Delayed in Matrix Metalloproteinase-9 and -12 Null Mice. *Journal of Neuroscience* **26**, 2207–2214 (2006).

174. Oh, L. Y. S. *et al.* Matrix Metalloproteinase-9/Gelatinase B Is Required for Process Outgrowth by Oligodendrocytes. *Journal of Neuroscience* **19**, 8464–8475 (1999).

175. Woo, M. S., Park, J. S., Choi, I. Y., Kimf, W. K. & Kim, H. S. Inhibition of MMP-3 or -9 suppresses lipopolysaccharide-induced expression of proinflammatory cytokines and iNOS in microglia. *J Neurochem* **106**, 770–780 (2008).

176. Lakhan, S. E., Kirchgessner, A., Tepper, D. & Leonard, A. Matrix Metalloproteinases and Blood-Brain Barrier Disruption in Acute Ischemic Stroke. *Front Neurol* **4**, (2013).

177. Dyck, S. M. & Karimi-Abdolrezaee, S. Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. *Exp Neurol* **269**, 169–187 (2015).

178. Morawski, M., Brückner, M. K., Riederer, P., Brückner, G. & Arendt, T. Perineuronal nets potentially protect against oxidative stress. *Exp Neurol* **188**, 309–315 (2004).

179. Miao, J. *et al.* Pleiotrophin promotes microglia proliferation and secretion of neurotrophic factors by activating extracellular signal-regulated kinase 1/2 pathway. *Neurosci Res* **74**, 269–276 (2012).

180. Wang, X. Pleiotrophin: Activity and mechanism. *Adv Clin Chem* (2020) doi:10.1016/bs.acc.2020.02.003.

181. Tang, C. *et al.* Neural Stem Cells Behave as a Functional Niche for the Maturation of Newborn Neurons through the Secretion of PTN. *Neuron* **101**, 32-44.e6 (2019).

182. del Olmo, N. *et al.* Pleiotrophin inhibits hippocampal long-term potentiation: A role of pleiotrophin in learning and memory. *Growth Factors* **27**, 189–194 (2009).

183. del Olmo, N. *et al.* Pleiotrophin inhibits hippocampal long-term potentiation: A role of pleiotrophin in learning and memory. *Growth Factors* **27**, 189–194 (2009).

184. Gombash, S. E. *et al.* Neuroprotective potential of pleiotrophin overexpression in the striatonigral pathway compared with overexpression in both the striatonigral and nigrostriatal pathways. *Gene Ther* **21**, 682 (2014).

185. Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H. & Noda, M. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase ζ/RPTPβ, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *Journal of Biological Chemistry* **271**, 21446–21452 (1996).

186. Perez-Pinera, P., Berenson, J. R. & Deuel, T. F. Pleiotrophin, a multifunctional angiogenic factor: Mechanisms and pathways in normal and pathological angiogenesis. *Curr Opin Hematol* **15**, 210–214 (2008).

187. Boese, A. C., Le, Q. S. E., Pham, D., Hamblin, M. H. & Lee, J. P. Neural stem cell therapy for subacute and chronic ischemic stroke. *Stem Cell Res Ther* **9**, 1–17 (2018).

188. Bang, O. Y., Kim, E. H., Cha, J. M. & Moon, G. J. Adult stem cell therapy for stroke: Challenges and progress. *J Stroke* **18**, 256–266 (2016).

189. Nikolakopoulou, A. M. *et al.* Pericyte loss leads to circulatory failure and pleiotrophin depletion causing neuron loss. *Nat Neurosci* **22**, 1089–1098 (2019).

190. Nikolakopoulou, A. M. *et al.* Pericyte loss leads to circulatory failure and pleiotrophin depletion causing neuron loss. *Nat Neurosci* **22**, 1089–1098 (2019).

191. Mi, R., Chen, W. & Ho, A. Pleiotrophin is a neurotrophic factor for spinal. *Spinal Cord* (2007) doi:10.1073/pnas.0603243104.

192. Yeh, H.-J., He, Y. Y., Xu, J., Hsu, C. Y. & Deuel, T. F. Upregulation of Pleiotrophin Gene Expression in Developing Microvasculature, Macrophages, and Astrocytes after Acute Ischemic Brain Injury. *The Journal of Neuroscience* **18**, 3699–3707 (2018).

193. Yanagisawa, H., Komuta, Y., Kawano, H., Toyoda, M. & Sango, K. Pleiotrophin induces neurite outgrowth and up-regulates growth-associated protein (GAP)-43 mRNA through the ALK/GSK3β/β-catenin signaling in developing mouse neurons. *Neurosci Res* **66**, 111–116 (2009).

194. Alsmadi, N. Z. *et al.* Glial-derived growth factor and pleiotrophin synergistically promote axonal regeneration in critical nerve injuries. *Acta Biomater* **78**, 165–177 (2018).

195. Paveliev, M. *et al.* HB-GAM (pleiotrophin) reverses inhibition of neural regeneration by the CNS extracellular matrix. *Sci Rep* **6**, 1–17 (2016).

196. Milev, P. *et al.* High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-ζ/β with tenascin-R, amphoterin, and the heparin-binding gowth-associated molecule. *Journal of Biological Chemistry* **273**, 6998–7005 (1998).

197. Fernández-Calle, R. *et al.* Pleiotrophin regulates microglia-mediated neuroinflammation. *J Neuroinflammation* **14**, 1–10 (2017).

198. Miao, J. *et al.* Pleiotrophin promotes microglia proliferation and secretion of neurotrophic factors by activating extracellular signal-regulated kinase 1/2 pathway. *Neurosci Res* **74**, 269–276 (2012).

199. Wiersma, A. M. Augmenting Plasticity and Recovery from Stroke by Modulating the Extracellular Matrix of the Central Nervous System.

200. Sim, F. J. *et al.* Complementary patterns of gene expression by human oligodendrocyte progenitors and their environment predict determinants of progenitor maintenance and differentiation. *Ann Neurol* **59**, 763–779 (2006).

201. McClain, C. R., Sim, F. J. & Goldman, S. A. Pleiotrophin Suppression of Receptor Protein Tyrosine Phosphatase- / Maintains the Self-Renewal Competence of Fetal Human Oligodendrocyte Progenitor Cells. *Journal of Neuroscience* vol. 32 15066–15075 Preprint at https://doi.org/10.1523/jneurosci.1320-12.2012 (2012).

202. Kuboyama, K., Fujikawa, A., Suzuki, R. & Noda, M. Inactivation of Protein Tyrosine Phosphatase Receptor Type Z by Pleiotrophin Promotes Remyelination through Activation of Differentiation of Oligodendrocyte Precursor Cells. *Journal of Neuroscience* **35**, 12162–12171 (2015).

203. Kuboyama, K., Fujikawa, A., Suzuki, R. & Noda, M. Inactivation of Protein Tyrosine Phosphatase Receptor Type Z by Pleiotrophin Promotes Remyelination through Activation of Differentiation of Oligodendrocyte Precursor Cells. *Journal of Neuroscience* **35**, 12162–12171 (2015).

204. Kuboyama, K., Tanga, N., Suzuki, R., Fujikawa, A. & Noda, M. Protamine neutralizes chondroitin sulfate proteoglycan-mediated inhibition of oligodendrocyte differentiation. *PLoS One* **12**, 1–21 (2017).

205. Warita, H. *et al.* Aberrant astrocytic expression of chondroitin sulfate proteoglycan receptors in a rat model of amyotrophic lateral sclerosis. *J Neurosci Res* **96**, 222–233 (2017).

206. Tang, C. *et al.* Neural Stem Cells Behave as a Functional Niche for the Maturation of Newborn Neurons through the Secretion of PTN. *Neuron* **101**, 32-44.e6 (2019).

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

208. Kuboyama, K., Fujikawa, A., Suzuki, R. & Noda, M. Inactivation of protein tyrosine phosphatase receptor type Z by pleiotrophin promotes remyelination through activation of differentiation of oligodendrocyte precursor cells. *Journal of Neuroscience* **35**, 12162–12171 (2015).

209. Yeh, H. J., He, Y. Y., Xu, J., Hsu, C. Y. & Deuel, T. F. Upregulation of pleiotrophin gene expression in developing microvasculature, macrophages, and astrocytes after acute ischemic brain injury. *Journal of Neuroscience* **18**, 3699–3707 (1998).

210. Fernández-Calle, R. *et al.* Pleiotrophin regulates microglia-mediated neuroinflammation. *J Neuroinflammation* **14**, 1–10 (2017).

211. Ridet, J. L., Malhotra, S. K., Privat, A. & Gage, F. H. Reactive astrocytes: Cellular and molecular cues to biological function. *Trends Neurosci* **20**, 570–577 (1997).

212. Anderson, M. F., Blomstrand, F., Blomstrand, C., Eriksson, P. S. & Nilsson, M. Astrocytes and stroke: Networking for survival? *Neurochem Res* **28**, 293–305 (2003).

213. Fawcett, J. W. & Asher, R. A. The glial scar and central nervous system repair. *Brain Res Bull* **49**, 377–391 (1999).

214. Morawski, M., Brückner, M. K., Riederer, P., Brückner, G. & Arendt, T. Perineuronal nets potentially protect against oxidative stress. *Exp Neurol* **188**, 309–315 (2004).

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

216. Wang, H. *et al.* Chondroitin-4-sulfation negatively regulates axonal guidance and growth. *J Cell Sci* **121**, 3083–3091 (2008).

217. Galtrey, C. M. & Fawcett, J. W. The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Res Rev* **54**, 1–18 (2007).

218. Herradõn, G. & Pérez-García, C. Targeting midkine and pleiotrophin signalling pathways in addiction and neurodegenerative disorders: Recent progress and perspectives. *British Journal of Pharmacology* vol. 171 837–848 Preprint at https://doi.org/10.1111/bph.12312 (2014).

219. Keough, M. B. *et al.* An inhibitor of chondroitin sulfate proteoglycan synthesis promotes central nervous system remyelination. *Nat Commun* **7**, 1–12 (2016).

220. Wong, J. W. J. Knocking down glycosaminoglycan synthesis. *Journal of Neuroscience* **28**, 2688–2689 (2008).

221. Dreyfus, J., Brunet-De Carvalho, N., Duprez, D., Raulais, D. & Vigny, M. HB-GAM/pleiotrophin: Localization of mRNA and protein in the chicken developing leg. *International Journal of Developmental Biology* **42**, 189–198 (1998).

222. Wang, X. Pleiotrophin: Activity and mechanism. *Adv Clin Chem* (2020) doi:10.1016/bs.acc.2020.02.003.

223. Tang, C. *et al.* Neural Stem Cells Behave as a Functional Niche for the Maturation of Newborn Neurons through the Secretion of PTN. *Neuron* **101**, 32-44.e6 (2019).

224. Bertram, S. *et al.* Pleiotrophin increases neurite length and number of spiral ganglion neurons in vitro. *Exp Brain Res* **237**, 2983–2993 (2019).

225. Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H. & Noda, M. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase ζ/RPTPβ, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *Journal of Biological Chemistry* **271**, 21446–21452 (1996).

226. Vernersson, E. *et al.* Characterization of the expression of the ALK receptor tyrosine kinase in mice. *Gene Expression Patterns* **6**, 448–461 (2006).

227. Iwahara, T. *et al.* Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* **14**, 439–449 (1997).

228. Yao, S. *et al.* Anaplastic Lymphoma Kinase Is Required for Neurogenesis in the Developing Central Nervous System of Zebrafish. *PLoS One* **8**, (2013).

229. Motegi, A., Fujimoto, J., Kotani Masaharu, Sakuraba Hitoshi & Yamamoto, T. ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. *J Cell Sci* **117**, 3319–3329 (2004).

230. Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nat Methods* **9**, 676–682 (2012).

231. Longair, M. H., Baker, D. A. & Armstrong, J. D. Simple neurite tracer: Open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* **27**, 2453–2454 (2011).

232. Herradon, G. & Ezquerra, L. Blocking Receptor Protein Tyrosine Phosphatase B/Z: A Potential Therapeutic Strategy for Parkinsons Disease. *Curr Med Chem* **16**, 3322–3329 (2009).

233. Santarpia, M., Altavilla, G. & Rosell, R. Alectinib: A selective, next-generation ALK inhibitor for treatment of ALK-rearranged non-small-cell lung cancer. *Expert Rev Respir Med* **9**, 255–268 (2015).

234. Alam, M. W. *et al.* Alectinib, an Anaplastic Lymphoma Kinase Inhibitor, Abolishes ALK Activity and Growth in ALK-Positive Neuroblastoma Cells. *Front Oncol* **9**, 21–23 (2019).

235. Beardslee, T. & Lawson, J. Alectinib and Brigatinib: New Second-Generation ALK Inhibitors for the Treatment of Non-Small Cell Lung Cancer. *J Adv Pract Oncol* **9**, 94–101 (2018).

236. Hida, H. *et al.* Pleiotrophin promotes functional recovery after neural transplantation in rats. *Neuroreport* **18**, 179–183 (2007).

237. Palmer, R. H., Vernersson, E., Grabbe, C. & Hallberg, B. Anaplastic lymphoma kinase: signalling in development and disease. *Biochemical Journal* **420**, 345–361 (2009).

238. Wulf, A. M., Moreno, M. M., Paka, C., Rampasekova, A. & Liu, K. J. Defining pathological activities of alk in neuroblastoma, a neural crest-derived cancer. *Int J Mol Sci* **22**, 1–20 (2021).

239. Miao, J. *et al.* Pleiotrophin promotes microglia proliferation and secretion of neurotrophic factors by activating extracellular signal-regulated kinase 1/2 pathway. *Neurosci Res* **74**, 269–276 (2012).

240. Fünfschilling, U. *et al.* Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* **485**, 517–521 (2012).

241. Kuboyama, K., Tanga, N., Suzuki, R., Fujikawa, A. & Noda, M. Protamine neutralizes chondroitin sulfate proteoglycan-mediated inhibition of oligodendrocyte differentiation. *PLoS One* **12**, 1–21 (2017).

242. Janowska, J., Ziemka-Nalecz, M. & Sypecka, J. The differentiation of rat oligodendroglial cells is highly influenced by the oxygen tension: In vitro model mimicking physiologically normoxic conditions. *Int J Mol Sci* **19**, 1–20 (2018).

243. Bergles, D. E. & Richardson, W. D. Oligodendrocyte development and plasticity. *Cold Spring Harb Perspect Biol* **8**, 1–28 (2016).

244. de Faria, O., Gonsalvez, D. G., Nicholson, M. & Xiao, J. Activity-dependent central nervous system myelination throughout life. *J Neurochem* **148**, 447–461 (2019).

245. Demerens, C. *et al.* Induction of myelination in the central nervous system by electrical activity. *Proc Natl Acad Sci U S A* **93**, 9887–9892 (1996).

246. de Faria, O., Gonsalvez, D. G., Nicholson, M. & Xiao, J. Activity-dependent central nervous system myelination throughout life. *J Neurochem* **148**, 447–461 (2019).

247. Barres, B. A. & Raff, M. C. *Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons*. *J. l. J. Phys,a/ .. Land* vol. 25.

248. Raffaele, S. & Fumagalli, M. Dynamics of Microglia Activation in the Ischemic Brain: Implications for Myelin Repair and Functional Recovery. *Front Cell Neurosci* **16**, 1–10 (2022).

249. Waly, B. El, Macchi, M., Cayre, M. & Durbec, P. Oligodendrogenesis in the normal and pathological central nervous system. *Front Neurosci* **8**, 1–22 (2014).

250. Lee, Y. *et al.* Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* **487**, 443–448 (2012).

251. Rauch, U. & Kappler, J. Chondroitin/Dermatan Sulfates in the Central Nervous System: Their Structures and Functions in Health and Disease. *Adv Pharmacol* **53**, 337–356 (2006).

252. Pu, A., Stephenson, E. L. & Yong, V. W. The extracellular matrix: Focus on oligodendrocyte biology and targeting CSPGs for remyelination therapies. *Glia* **66**, 1809–1825 (2018).

253. Fawcett, J. W. & Asher, R. A. The glial scar and central nervous system repair. *Brain Res Bull* **49**, 377–391 (1999).

254. Lau, L. W., Cua, R., Keough, M. B., Haylock-Jacobs, S. & Yong, V. W. Pathophysiology of the brain extracellular matrix: A new target for remyelination. *Nat Rev Neurosci* **14**, 722–729 (2013).

255. Sun, Y. *et al.* Chondroitin sulfate proteoglycans inhibit the migration and differentiation of oligodendrocyte precursor cells and its counteractive interaction with laminin. *Int J Mol Med* **40**, 1657–1668 (2017).

256. Dyck, S. *et al.* Perturbing chondroitin sulfate proteoglycan signaling through LAR and PTPσ receptors promotes a beneficial inflammatory response following spinal cord injury. *J Neuroinflammation* **15**, 1–26 (2018).

257. Keough, M. B. *et al.* Overcoming neurite-inhibitory chondroitin sulfate proteoglycans in the astrocyte matrix. *Glia* **61**, 972–984 (2013).

258. Stephenson, E. L. & Yong, V. W. Pro-inflammatory roles of chondroitin sulfate proteoglycans in disorders of the central nervous system. *Matrix Biology* **71–72**, 432–442 (2018).

259. L M Matrisian. The matrix-degrading metalloproteinases. *BioEssay* **153**, 545–548 (1962).

260. Gorter, R. P. & Baron, W. Matrix metalloproteinases shape the oligodendrocyte (niche) during development and upon demyelination. *Neurosci Lett* **729**, 134980 (2020).

261. Lau, L. W. *et al.* Chondroitin sulfate proteoglycans in demyelinated lesions impair remyelination. *Ann Neurol* **72**, 419–432 (2012).

262. Pu, A., Stephenson, E. L. & Yong, V. W. The extracellular matrix: Focus on oligodendrocyte biology and targeting CSPGs for remyelination therapies. *Glia* **66**, 1809–1825 (2018).

263. Šišková, Z. *et al.* Fibronectin attenuates process outgrowth in oligodendrocytes by mislocalizing MMP-9 activity. *Molecular and Cellular Neuroscience* **42**, 234–242 (2009).

264. Gorter, R. P. & Baron, W. Matrix metalloproteinases shape the oligodendrocyte (niche) during development and upon demyelination. *Neurosci Lett* **729**, (2020).

265. Rolls, A. *et al.* Two faces of chondroitin sulfate proteoglycan in spinal cord repair: A role in microglia/ macrophage activation. *PLoS Med* **5**, 1262–1277 (2008).

266. Könnecke, H. & Bechmann, I. The role of microglia and matrix metalloproteinases involvement in neuroinflammation and gliomas. *Clin Dev Immunol* **2013**, (2013).

267. Tanga, N. *et al.* The PTN-PTPRZ signal activates the AFAP1L2-dependent PI3K-AKT pathway for oligodendrocyte differentiation: Targeted inactivation of PTPRZ activity in mice. *Glia* **67**, 967–984 (2019).

268. McClain, C. R., Sim, F. J. & Goldman, S. A. Pleiotrophin Suppression of Receptor Protein Tyrosine Phosphatase- / Maintains the Self-Renewal Competence of Fetal Human Oligodendrocyte Progenitor Cells. *Journal of Neuroscience* vol. 32 15066–15075 Preprint at https://doi.org/10.1523/jneurosci.1320-12.2012 (2012).

269. Cunha, M. I. *et al.* Pro-inflammatory activation following demyelination is required for myelin clearance and oligodendrogenesis. *Journal of Experimental Medicine* **217**, (2020).

270. Fernández-Calle, R. *et al.* Pleiotrophin regulates microglia-mediated neuroinflammation. *J Neuroinflammation* **14**, 1–10 (2017).

271. Tiago A Ferreira, Arne V Blackman, Julia Oyrer, Sriram Jayabal, Andrew J Chung, Alanna J Watt, P. J. S. & D. J. van M. Neuronal morphometry directly from bitmap images. *Nat Methods* **11**, 981–982 (2014).

272. Rosenberg, G. A. *et al.* Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: Activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain Res* **893**, 104–112 (2001).

273. Hsu, J. Y. C. *et al.* Matrix metalloproteinase-2 facilitates wound healing events that promote functional recovery after spinal cord injury. *Journal of Neuroscience* **26**, 9841–9850 (2006).

274. Ma, Z., Qin, H. & Benveniste, E. N. Transcriptional Suppression of Matrix Metalloproteinase-9 Gene Expression by IFN-γ and IFN-β: Critical Role of STAT-1α. *The Journal of Immunology* **167**, 5150–5159 (2001).

275. Dyck, S. M. & Karimi-Abdolrezaee, S. Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. *Exp Neurol* **269**, 169–187 (2015).

276. Kuboyama, K., Fujikawa, A., Suzuki, R. & Noda, M. Inactivation of protein tyrosine phosphatase receptor type Z by pleiotrophin promotes remyelination through activation of differentiation of oligodendrocyte precursor cells. *Journal of Neuroscience* **35**, 12162–12171 (2015).

277. Tanga, N. *et al.* The PTN-PTPRZ signal activates the AFAP1L2-dependent PI3K-AKT pathway for oligodendrocyte differentiation: Targeted inactivation of PTPRZ activity in mice. *Glia* **67**, 967–984 (2019).

278. Lin, W. *et al.* Interferon-γ inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress. *Brain* **129**, 1306–1318 (2006).

279. Harrington, E. P., Bergles, D. E. & Calabresi, P. A. Immune cell modulation of oligodendrocyte lineage cells. *Neurosci Lett* **715**, 134601 (2020).

280. Porro, C., Cianciulli, A. & Panaro, M. A. The Regulatory Role of IL-10 in Neurodegenerative Diseases. *Biomolecules* **10**, 1–15 (2020).

281. Laffer, B. *et al.* Loss of IL-10 Promotes Differentiation of Microglia to a M1 Phenotype. *Front Cell Neurosci* **13**, 473875 (2019).

282. Anrather, J. & Iadecola, C. Inflammation and Stroke: An Overview. *Neurotherapeutics* **13**, 661–670 (2016).

283. Seifert, H. A. *et al.* Pro-inflammatory interferon gamma signaling is directly associated with stroke induced neurodegeneration. *J Neuroimmune Pharmacol* **9**, 679–689 (2014).

284. Yilmaz, G., Arumugam, T. V., Stokes, K. Y. & Granger, D. N. Role of T Lymphocytes and Interferon-γ in Ischemic Stroke. *Circulation* **113**, 2105–2112 (2006).

285. Fernández-Calle, R. *et al.* Pleiotrophin regulates microglia-mediated neuroinflammation. *J Neuroinflammation* **14**, 1–10 (2017).

286. Ginhoux, F. & Prinz, M. Origin of microglia: Current concepts and past controversies. *Cold Spring Harb Perspect Biol* **7**, 1–15 (2015).

287. Ginhoux, F., Lim, S., Hoeffel, G., Low, D. & Huber, T. Origin and differentiation of microglia. *Front Cell Neurosci* **7**, 1–14 (2013).

288. Ilia D. Vainchtein, A. V. M. Astrocytes and microglia: in sickness and in health Ilia. *Trends Neurosci.* **176**, 139–148 (2020).

289. Laabs, T., Carulli, D., Geller, H. M. & Fawcett, J. W. Chondroitin sulfate proteoglycans in neural development and regeneration. *Curr Opin Neurobiol* **15**, 116–120 (2005).

290. Pearson, C. S., Mencio, C. P., Barber, A. C., Martin, K. R. & Geller, H. M. Identification of a critical sulfation in chondroitin that inhibits axonal regeneration. *Elife* **7**, 1–21 (2018).

291. Perez-Pinera, P., Berenson, J. R. & Deuel, T. F. Pleiotrophin, a multifunctional angiogenic factor: Mechanisms and pathways in normal and pathological angiogenesis. *Curr Opin Hematol* **15**, 210–214 (2008).

292. Yin, K. J. *et al.* Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism. *Journal of Neuroscience* vol. 26 10939–10948 Preprint at https://doi.org/10.1523/JNEUROSCI.2085-06.2006 (2006).

293. Constantinescu, C. S. *et al.* Astrocytes as antigen-presenting cells: Expression of IL-12/IL-23. *J Neurochem* **95**, 331–340 (2005).

294. Rothhammer, V. & Quintana, F. J. Control of autoimmune CNS inflammation by astrocytes. *Semin Immunopathol* **37**, 625–638 (2015).

295. Dong, Y. & Benveniste, E. N. Immune function of astrocytes. *Glia* **36**, 180–190 (2001).

296. Lau, L. T. & Yu, A. C. H. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *J Neurotrauma* **18**, 351–359 (2001).

297. Sterpka, A. *et al.* Diverged morphology changes of astrocytic and neuronal primary cilia under reactive insults. *Mol Brain* **13**, 1–16 (2020).

298. Linnerbauer, M. *et al.* Astrocyte-Derived Pleiotrophin Mitigates Late-Stage Autoimmune CNS Inflammation. *Front Immunol* **12**, 1–14 (2022).

299. Matejuk, A. & Ransohoff, R. M. Crosstalk Between Astrocytes and Microglia: An Overview. *Front Immunol* **11**, 1–11 (2020).

300. Ding, X. *et al.* Silencing IFN-γ Binding/Signaling in Astrocytes versus Microglia Leads to Opposite Effects on Central Nervous System Autoimmunity. *The Journal of Immunology* **194**, 4251–4264 (2015).

301. Pradip K Kamat1, Supriya Swarnkar2, Shivika Rai3, Vijay Kumar4, and N. T. Astrocyte mediated MMP-9 activation in the synapse dysfunction: An implication in Alzheimer disease Pradip. *Bone* **23**, 1–7 (2008).

302. Cabral-Pacheco, G. A. *et al.* The roles of matrix metalloproteinases and their inhibitors in human diseases. *Int J Mol Sci* **21**, 1–53 (2020).

303. Brew, K. & Nagase, H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta* **1803**, 55 (2010).

304. Welser-Alves, J. V., Crocker, S. J. & Milner, R. A dual role for microglia in promoting tissue inhibitor of metalloproteinase (TIMP) expression in glial cells in response to neuroinflammatory stimuli. *J Neuroinflammation* **8**, 61 (2011).

305. Estlmates, D. Stroke Disability. *Phys Ther* **74**, (1994).

306. Cramer, S. C. & Nudo, R. J. Brain repair after stroke. *Brain Repair After Stroke* 1–296 (2010) doi:10.1017/CBO9780511777547.

307. Katan, M. & Luft, A. Global Burden of Stroke. *Semin Neurol* **38**, 208–211 (2018).

308. Mergenthaler, P., Dirnagl, U. & Kunz, A. Ischemic Stroke: Basic Pathophysiology and Clinical Implication. in *Neuroscience in the 21st Century: From Basic to Clinical* (ed. Pfaff, D. W.) 2543–2563 (Springer New York, 2013). doi:10.1007/978-1-4614-1997-6_97.

309. Ming, G. li & Song, H. Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. *Neuron* **70**, 687 (2011).

310. Cameron, H. A. & Glover, L. R. Adult Neurogenesis: Beyond Learning and Memory*. *https://doi.org/10.1146/annurev-psych-010814-015006* **66**, 53–81 (2015).

311. Carmichael, S. T., Kathirvelu, B., Schweppe, C. A. & Nie, E. H. Molecular, cellular and functional events in axonal sprouting after stroke. *Experimental Neurology* vol. 287 384–394 Preprint at https://doi.org/10.1016/j.expneurol.2016.02.007 (2017).

312. Lindvall, O. & Kokaia, Z. Neurogenesis following Stroke Affecting the Adult Brain. *Cold Spring Harb Perspect Biol* **7**, (2015).

313. Ohab, J. J., Fleming, S., Blesch, A. & Carmichael, S. T. A neurovascular niche for neurogenesis after stroke. *J Neurosci* **26**, 13007–13016 (2006).

314. Carmichael, S. T., Kathirvelu, B., Schweppe, C. A. & Nie, E. H. Molecular, cellular and functional events in axonal sprouting after stroke. *Exp Neurol* **287**, 384–394 (2017).

315. Marin, M. A. & Carmichael, S. T. Stroke in CNS white matter: Models and mechanisms. *Neuroscience Letters* vol. 684 193–199 Preprint at https://doi.org/10.1016/j.neulet.2018.07.039 (2018).

316. Chen, D. *et al.* Demyelinating processes in aging and stroke in the central nervous system and the prospect of treatment strategy. *CNS Neurosci Ther* **26**, 1219 (2020).

317. Yenari, M. A., Kauppinen, T. M. & Swanson, R. A. Microglial Activation in Stroke: Therapeutic Targets. *Neurotherapeutics* **7**, 378–391 (2010).

318. DeFrancesco-Lisowitz, A., Lindborg, J. A., Niemi, J. P. & Zigmond, R. E. The Neuroimmunology of Degeneration and Regeneration in the Peripheral Nervous System. *Neuroscience* **302**, 174 (2015).

319. Taylor, R. A. & Sansing, L. H. Microglial responses after ischemic stroke and intracerebral hemorrhage. *Clin Dev Immunol* **2013**, (2013).

320. Qin, C. *et al.* Dual Functions of Microglia in Ischemic Stroke. *Neurosci Bull* **35**, 921–933 (2019).

321. Li-Jin Chew, Asako Takanohashi, and M. B. MICROGLIA AND INFLAMMATION:IMPACT ON DEVELOPMENTAL BRAIN INJURIES. **287**, 280–287 (2006).

322. Li, H., Ghorbani, S., Ling, C. C., Yong, V. W. & Xue, M. The extracellular matrix as modifier of neuroinflammation and recovery in ischemic stroke and intracerebral hemorrhage. *Neurobiol Dis* **186**, 106282 (2023).

323. Walberer, M. *et al.* In-vivo detection of inflammation and neurodegeneration in the chronic phase after permanent embolic stroke in rats. *Brain Res* **1581**, 80–88 (2014).

324. Wang, Y., Leak, R. K. & Cao, G. Microglia-mediated neuroinflammation and neuroplasticity after stroke. *Front Cell Neurosci* **16**, (2022).

325. Fang, J., Wang, Z. & Miao, C. yu. Angiogenesis after ischemic stroke. *Acta Pharmacologica Sinica 2023 44:7* **44**, 1305–1321 (2023).

326. Navaratna, D., Guo, S., Arai, K. & Lo, E. H. Mechanisms and targets for angiogenic therapy after stroke. *Cell Adh Migr* **3**, 216 (2009).

327. Huang, L. *et al.* Glial scar formation occurs in the human brain after ischemic stroke. *Int J Med Sci* **11**, 344–348 (2014).

328. Ohtake, Y. & Li, S. Molecular mechanisms of scar-sourced axon growth inhibitors. *Brain Res* **1619**, 22 (2015).

329. Dewang, P., Hsu, N.-M., Peng, S.-Z. & Li, W.-R. Protein tyrosine phosphatases and their inhibitors. *Curr Med Chem* **12**, 1–22 (2005).

330. Asai, H., Morita, S. & Miyata, S. Effect of pleiotrophin on glutamate-induced neurotoxicity in cultured hippocampal neurons. *Cell Biochem Funct* **29**, 660–665 (2011).

331. Gonsalvez, D. G. *et al.* Imaging and Quantification of Myelin Integrity After Injury With Spectral Confocal Reflectance Microscopy. *Front Mol Neurosci* **12**, 478757 (2019).