2023-09-20

Repurposing repeated remote ischemic postconditioning for multiple sclerosis

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Repurposing repeated remote ischemic postconditioning for multiple sclerosis

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN NEUROSCIENCE

CALGARY, ALBERTA

SEPTEMBER, 2023

Abstract

Multiple sclerosis (MS) is a chronic, autoimmune neurodegenerative disease that is characterized by nervous system demyelination and heterogenous disability. Current disease modifying treatments accessible to Canadians are limited because they are primarily targeting the neuroinflammatory component of the disease, not the neurodegenerative component. There is a need for focus on development of therapies that target the demyelinating insults that people with MS experience. In this study, we evaluate the use of repeated remote ischemic postconditioning (RIC) as a therapeutic target for promoting white matter repair and protection. Due to the novelty of the intervention in the MS field, we first wanted to identify transcriptomic and proteomic changes to the spinal cord with repeated RIC. We found that targets involved in antioxidant, protein synthesis, angiogenesis, axonogenesis, and remyelination pathways (among others) were upregulated with 14 days of consecutive RIC. Using a focal demyelinating mouse model, we also described changes to the lesion environment using the repeated intervention. Repeated RIC did not reduce the lesion size in this injury model. However, targets such as plectin and neurofascin that were upregulated in the transcriptomic and proteomic data sets were also upregulated in the lesion of animals that received treatment. An increase in myelin in the lesion area was also found in the repeated RIC group when compared to sham. Overall, repeated RIC demonstrates potential for future use as a therapy to target white matter repair and protection.

Keywords: multiple sclerosis, remyelination, remote ischemic conditioning, remote ischemic postconditioning, hypoxia, oligodendrocyte, oligodendrocyte precursor cell, neuroprotection, neurorepair

Preface

This thesis is an original and unpublished work by the author. The study was granted ethics approval by the Health Sciences Animal Care Committee (HSACC) under the study #AC20-0061 “Mechanisms of Remyelination in the CNS” on June 29th, 2020.

Acknowledgments

First, I offer my sincere appreciation and gratitude to my supervisor Dr. Carlos Camara-Lemarroy. Thank you for trusting me as your first graduate student. Over the past three years, you have provided me with invaluable knowledge and development that will prevail throughout my career and lifetime. Thank you for giving me unwavering support during the successes and failures of my project. Through every challenge I faced, you offered expertise and guidance that allowed me to solve everything with a considerable level of independence. Your intelligence, clinical insight, and passion for research is something to be admired. You have been an incredible mentor, and I know that the students after me will be lucky to have you as a supervisor. I look forward to witnessing your incredible academic and clinical career unfold.

Second, I express gratitude to my supervisory committee: Dr. V. Wee Yong, Dr. Shalina Ousman, and Dr. Eric E. Smith. Thank you for supporting me during the progress of my program. You have challenged me towards becoming a better researcher. I hold very high respect for your impact that you have had within your respective careers. I appreciate the precious time that you have spent giving me your attention, consideration and feedback. This project would not have been successful without your guidance. I would also like to thank my examiners, Dr. Alex Lohman and Dr. Andrew Braun, for taking time to participate in my final examination. Your feedback and consideration were appreciated wholeheartedly.

Third, thank you to my loved ones who have supported me through the most challenging, yet rewarding years to date. Dad, you have shown me unconditional love and support throughout my entire life. In academia, certainty can be a tough concept to demonstrate. If you're only going

to take one thing away from this thesis, know that I am certain that I would not be where I am today without you. There is no amount of gratitude that can come close to paying back what you have given me. You have been an incredible role model and parent. I can proudly say that my work ethic is attributed to you. I am so lucky to have you in my corner. Hannah Tottenham, Jennifer Tottenham, Emily Parsons, Avery Russell, Brooke Stoilen, Lauren Emerson, Nina Vitkova, Zachary Bailey: thank you for being the best cross-Canada support crew that I could have ever been blessed with. You have supported me with unwavering (and at times, undeserving) loyalty. I love and appreciate each of you. Finally, this wouldn't be an authentic familial acknowledgment without a final, special mention of appreciation to my four-legged supporters, Benson and Bonita. I love you both so very much.

Lastly, this thesis would not be successful without the guidance, support and advice from many additional people and organizations. In no order, thank you to Emily Wuerch, Dorsa Moezzi, Dr. Brian Lozinski, Dr. Jeff Dong, Claudia Silva, Bev Clair, the entire Yong lab, Dr. Antoine Dufour, Maggie Chopra, Dr. Paul Gordon, the Alberta Multiple Sclerosis Network, the HBI AMP facility and staff, the University of Calgary Faculty of Graduate Studies, and the Hotchkiss Brain Institute.

To Danton, in another lifetime…

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Abbreviations

BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycan
DMT(s)	Disease-modifying treatment(s)
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EM	Electron microscopy
GO	Gene ontology
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1 alpha
HSP	Heat shock protein
IgG	Immunoglobulin G
LPC	Lysophosphatidylcholine
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NAWM	Normal appearing white matter
Nfasc	Neurofascin
NF-κB	Nuclear factor-kappa B
NG2	Neural/glial antigen 2
OL(s)	Oligodendrocyte(s)

OPC(s)	Oligodendrocyte precursor cell(s)
PHD	Prolylhydroxylase
PLP	Myelin proteolipid protein
PPMS	Primary progressive multiple sclerosis
pwMS	People with multiple sclerosis
RCT	Randomized clinical trial
RIC	Remote ischemic conditioning
RIPerC	Remote ischemic perconditioning
RIPostC	Remote ischemic postconditioning
RIPreC	Remote ischemic preconditioning
RNA	Ribonucleic acid
ROI	Region of interest
RRMS	Relapsing-remitting multiple sclerosis
SD	Standard deviation
SPMS	Secondary progressive multiple sclerosis
VEGF	Vascular endothelial growth factor

A proclivity for science is embedded deeply within us, in all times, places, and cultures (...) At the heart of science is an essential balance between two seemingly contradictory attitudes -- an openness to new ideas, no matter how bizarre or counterintuitive they may be, and the most ruthless skeptical scrutiny of all ideas, old and new.

-Carl Sagan, *The Demon-Haunted World: Science as a Candle in the Dark*

Chapter 1: Introduction

1.1 Multiple Sclerosis

1.1.1 Disease Overview

Multiple sclerosis (MS) is a chronic, autoimmune disease that causes degeneration of the central nervous system. Globally, 2.4 million people live with MS (MS International Federation, 2022). Canada has one of the highest incidences of MS, with over 100 000 people diagnosed (MS Society of Canada). MS affects females more than males at a ratio of 3:1. However, males tend to experience the disease with increased severity of progression and prognosis (Walton et al., 2020). MS is a relatively heterogenous disease that can present differently based on clinical presentation, symptomology, and treatment response. MS can be diagnosed by a magnetic resonance imaging (MRI) scan that shows lesions within the central nervous system (CNS). The presence of a lesion is identified by dissemination in time and space involving multiple regions and at different points in time. Clinical measures are used in adjunct to confirm the diagnosis of MS. For example, the presence of oligoclonal bands and quantitative IgG index are two indicators of MS (Ömerhoca et al., 2018). The heterogeneity of the clinical course of MS has revealed that there are multiple subtypes of MS: clinically isolated syndrome (CIS), relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS). However, it is likely that MS exists on a disease spectrum as opposed to definitive subtypes. This is demonstrated in adjunct to the emerging concepts of personalized treatment and medicine in the field. (Antel et al., 2012; De Jager, 2009). RRMS is the most common form of MS, affecting approximately 85% of people with MS (pwMS) (Klineova & Lublin, 2018). RRMS is characterized by periods of disease remission, with intervals of disease activity that predominantly originate from a dysfunctional immune response. Over time, the disease can

develop into a progressive form. SPMS is characterized by increased neurodegeneration compared to neuroinflammation. From a cellular perspective, there is more myelin debris, increased meningeal inflammation and reduced capacity for remyelination in the progressive forms of the disease (Faissner et al., 2019). PPMS is another MS subtype which occurs when the person's first clinical presentation starts with progressive accumulation of disability. PPMS takes a harsh, neurodegenerative disease course with no periods of remission (Miller & Leary, 2007). It is not clear why some pwMS begin with RRMS while others are initially diagnosed as PPMS. It is also not fully understood why patients may progress from RRMS to progressive forms of the disease either. Further understanding of the underlying pathophysiological mechanisms of the disease is required.

Inflammatory episodes, immune system dysregulation, demyelination and subsequent axonal degeneration are hallmarks of MS pathophysiology. Neurological symptoms can present in different combinations of fatigue, chronic pain, vision impairment, bladder dysfunction, mobility limitation and cognitive dysfunction (Ghasemi et al., 2017). The variety of symptoms can stem from the heterogeneity of lesion localization of each person. Disease-modifying treatments (DMTs) are the primary suggestion for treatment of MS (McGinley et al., 2021). Other lifestyle factors such as change in diet, increased vitamin intake, and reducing smoking are also suggested by physicians. DMTs offered to RRMS patients decrease the frequency of relapses by targeting CNS immune cells. Despite efforts from researchers towards the promotion of remyelination, the majority of the currently available DMTs target the neuroimmune aspect of the disease (McGinley et al., 2021). There are not as many treatment options for pwMS that are experiencing the neurodegenerative aspect of the disease described in SPMS and PPMS. There is

an unmet need for accessible therapies available to pwMS that target repair or offer neuroprotection.

1.1.2 Cellular Pathophysiology of MS

MS displays both a neuroimmune and neurodegenerative component. Both neurodegeneration and pathological neuroimmunology contribute to the pathogenesis of MS in concert *and* independently, over time. Earlier stages of the disease are characterized by increased immune infiltration, while progressive forms of the disease tend to have more neurodegenerative character (Faissner et al., 2019). This section will provide a simplified overview of the cellular pathophysiology of MS.

Inflammation due to MS pathophysiology leads to demyelination in the nervous system. CNS inflammation in MS is thought to be caused by immune cell infiltration and subsequent exacerbation of immune-mediated damage to both gray and white matter tissues. A significant mediator of this initial infiltration is executed by CD4+ T cells (T helper/Th) cells from the adaptive immune system. These autoreactive T cells recognize CNS-specific proteins and induce infiltration and subsequent demyelination (Agrawal & Yong, 2007). Autoreactive T cells bound to myelin can lead to activation of macrophages and microglia for initiating myelin destruction via phagocytosis (Brück, 2005). Simultaneously, pathogen-associated molecules bind to toll-like receptors on antigen presenting cells, which leads to production of inflammatory cytokines such as TNF-α, IL-4, IL-12, and IL-23. Upregulation of these cytokines induces the expression of endothelial cell surface markers which facilitate tethering and rolling along the endothelial cell surface. T cells will bind to the endothelial cell surface using these markers and transmigrate through the endothelial layer (van Langelaar et al., 2020). Proinflammatory cytokines also

promote Th cell differentiation into phenotypes such as Th1, Th2 or Th17. Each Th cell phenotypes secrete a unique profile of cytokines. IFNγ is secreted by Th1 cells to activate macrophages, suppress Th2 proliferation and produce antibodies (Kunkl et al., 2020). Th2 cells secrete regulatory cytokines such as IL-4 and IL-13. Th17 cells produce Il-17 and IL-23 which direct migration and trafficking of neutrophils. In the EAE animal model and clinical research, Th1 cells play a role in the development of the disease while Th2 cells may be important for recovery (Oreja-Guevara et al., 2012). T cells may also release proinflammatory and cytotoxic cytokines that promote demyelination. For example, CD8+ T cells recognize and target oligodendrocyte and myelin antigens during inflammation (Höftberger et al., 2004). For many years, it was believed that autoreactive T cells were the only factor in development and progression of MS pathogenesis. This notion no longer holds merit and over the past decades, different cell types such as microglia/macrophages, regulatory T cells, B lymphocytes and natural killer (NK) cells are also important components that affect MS pathogenesis (Beliën et al., 2022; Danikowski et al., 2017; Jain & Yong, 2022).

Microglia and macrophages are immune surveillant cells that are important in understanding the pathophysiology of MS. The role of these cells is very multi-faceted, and the following information is a very broad overview of the roles that they play in MS. Macrophages can be categorized into an M1 or M2 phenotype (Luo et al., 2017). The M1 phenotype is typically associated with inflammation and oligodendrocyte damage, while the M2 phenotype is associated with immune modulation and repair. Microglia/macrophages are found in MS lesions, play a role in oxidative stress and remyelination, and signal other immune cells. Microglia/macrophages clear myelin debris via phagocytosis. This process also initiates further OPC recruitment and subsequent attempt at repair (Vogel et al., 2013). Different subgroups of

microglia/macrophages may play different roles in the context of MS. Many researchers have hypothesized that inducing a greater M2 phenotype in these cells may be beneficial for treatment of pwMS.

The cellular pathophysiology of MS can also be attributed to a multitude of other immune cells. B cells may be found in ventricles and perivascular space in MS (Comi et al., 2021). Proinflammatory CD20+ B cells are often found in early, active lesions. Infiltrating B cells release antibodies that target myelin. Release of cytotoxic mediators and free radicals from immune cells induce further oxidative stress (Lubetzki & Stankoff, 2014). Normal physiological function of regulatory T cells is primarily to maintain immune tolerance (Danikowski et al., 2017). In MS, it is thought that there is a regulatory T cell dysfunction that is not allowing the cell to mitigate the autoreactive T cells. pwMS have decreased regulatory T cell suppressive function and increased expression of apoptotic markers in the brain (Mastorodemos et al., 2014). NK cells are a lymphocyte that is part of the innate immune system, playing a role in fighting viral infections. In MS, it was found that NK cell subsets may play a role in the pathogenesis of the disease (Mimpen et al., 2020). NK cells can be cytotoxic and are found in pwMS CSF (Beliën et al., 2022). There are many complex cellular interactions that contribute to the pathophysiology of MS, some of which have not yet been described in research.

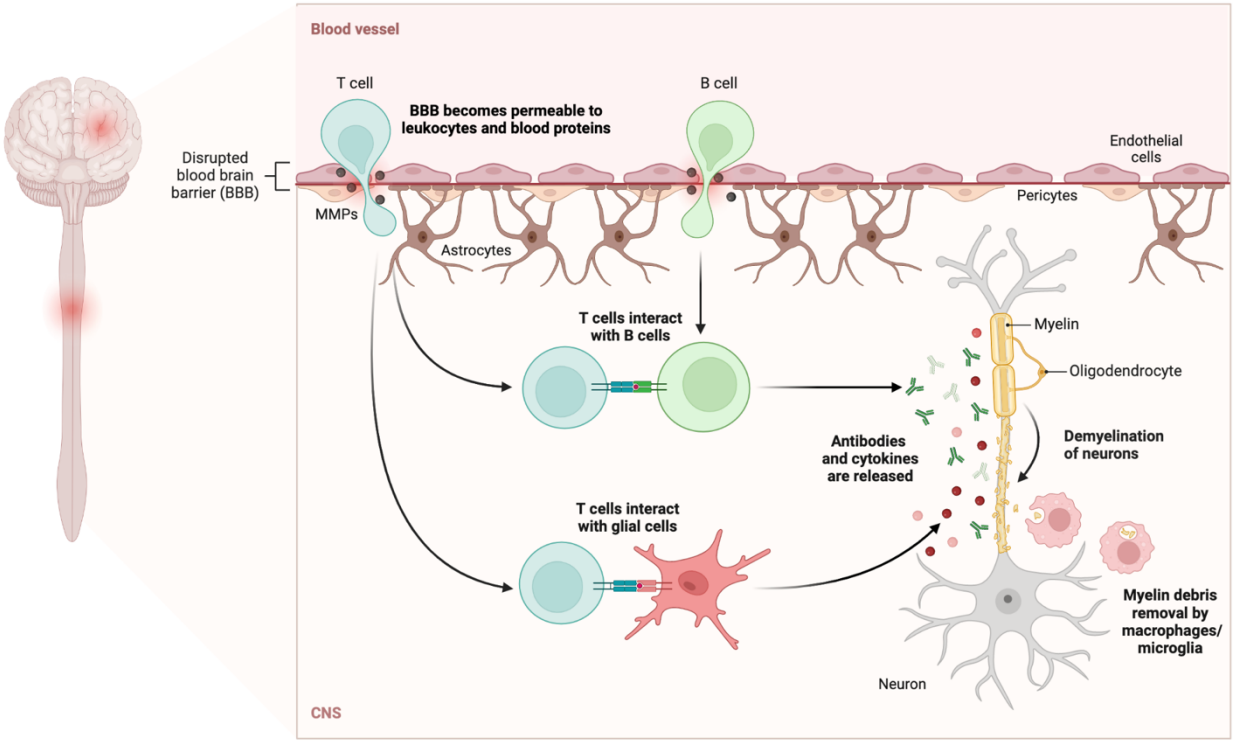


Figure 1: Overview of disease pathogenesis in multiple sclerosis. Simplified graphic of the cellular interactions that contribute to the pathogenesis of MS. Created with Biorender.

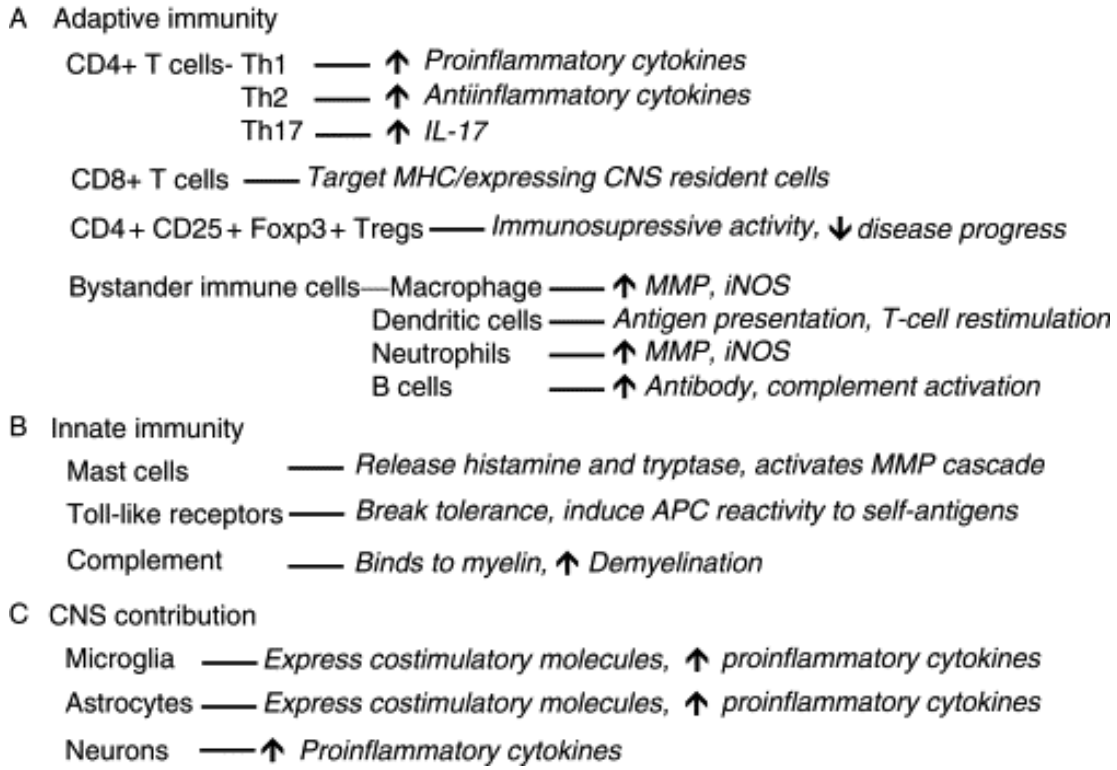


Figure 2: Cellular contributions to multiple sclerosis. Cells from the adaptive and innate immune system contribute to MS disease progression (from Agrawal & Yong, 2007).

1.2 Remyelination

1.2.1 Myelin Biology

Nervous system axons are protected by a fatty, insulating substance called myelin which is essential for signal conduction and proper cellular function (Bolino, 2021). Myelinated fibres allow for rapid transmission of signalling, called saltatory conduction, and maintenance of axonal integrity. Saltatory conduction describes the cluster of sodium channels at intervals called nodes of Ranvier. These clusters allow for a transmission "jump" from one node to the next. Conversely, unmyelinated axons have evenly distributed sodium channels and lack the ability to propagate their signals through this jumping action. In physiological condition, myelin is produced by myelinating oligodendrocytes in the CNS and Schwann cells in the peripheral

nervous system. OLs also provide trophic support and energy through provisions of nutrients. Lactate is an organic acid that has been demonstrated as an essential nutrient source for axons (Fünfschilling et al., 2012). OLs may also provide trophic support through the release of growth factors, such as insulin growth factor-1 (Wilkins et al., 2001). Experimentally-induced death of myelinating OLs has been associated with axonal damage and reduced remyelination (Pohl et al., 2011).

During remyelination in the adult CNS, it is unclear where new myelin originates. Prior to the discovery of oligodendrocyte precursor cells (OPCs), the two hypotheses were: 1) myelin sheaths were produced by new OLs or 2) OLs that survived the demyelinating insult contribute to the remyelination. The first hypothesis was further supported by the discovery of O-2A cells in 1983, which would later be called OPCs (Raff et al., 1983). OPCs are a precursor to mature OLs and are essential for remyelination in development and pathophysiology. It was recently shown that in a model of focal spinal cord injury, the pre-existing mature OLs did not contribute to remyelination of the injury (Crawford et al., 2016). This was accomplished with a transgenic mouse line that expressed a tamoxifen-inducible form of Cre recombinase in different OL lineage markers under transcriptional control. They concluded that pre-existing mature OLs did not migrate towards the injury during recruitment or extend into the lesion core from the periphery. In a contrasting study, it was shown that mature, pre-existing oligodendrocytes contributed to the remyelination in other animal models (Duncan et al., 2018). Evidence for both arguments have been supported in the literature. It can be postulated that pre-existing mature OLs may contribute to some, but not all, remyelination efforts after an injury.

During development, OPCs originate from neural stem cells that line ventricles. After their production, OPCs migrate away from the ventricles. Attractive and repellant cues guide OPC

migration. Examples of some of these cues include semaphorins, sonic hedgehog and netrin 1 (De Castro et al., 2013). Oligodendrogenesis follows a ventral-to-dorsal and caudal-to-rostral gradient during normal human development. Once the migrating OPCs reach their end destination, they begin to mature and express lineage markers such as myelin basic protein (MBP) and proteolipid protein (PLP) (Ruskamo et al., 2022). Typically, these markers indicate that the mature OL can myelinate surrounding axons in development and after a demyelinating injury.

There are two types of myelin degeneration: primary and secondary. Primary demyelination occurs when there is myelin loss occurring from an intact axon. Secondary demyelination, also known as Wallerian degeneration, occurs when the axon is injured, and subsequent demyelination occurs (Conforti et al., 2014). Upon demyelination, conduction through the nerve is impaired which can translate to functional loss of the neuronal pathway. One of the adaptive responses to reverse the impairment is the insertion of additional Na+ channels along the demyelinated axon to restore conduction (Franklin & Ffrench-Constant, 2008). The process of physiological remyelination in the CNS is characterized primarily by the activation, recruitment, and differentiation of OPCs. OPCs that express proteoglycan neuron-glial antigen 2 (NG2) are abundant in the adult CNS and are the precursor cells to mature, myelinating oligodendrocytes. Following a demyelinating insult, OPCs upregulate key transcription factors such as Olig2 (Fancy et al., 2004) and Sox2 (Zhao et al., 2015). Growth factors that are released locally are proposed to promote the recruitment of OPCs to the lesion area. Once recruited, OPCs undergo differentiation and maturation into mature OLs. OL express specific proteins and factors that help in the process of remyelination, like MBP.

Remyelinated axons can typically be distinguished post-injury because the myelin is thinner and shorter than developmental myelin production. However, it has been argued in an animal model that newly formed myelin derived from Schwann cells and oligodendrocytes developed over longer periods of time match baseline myelin characteristics (Powers et al., 2013). Remyelination occurs efficiently in a multitude of different injuries and traumas. Animal models such as cuprizone and LPC-induced demyelination demonstrate a well characterized sequence of demyelination followed by remyelination. There are certain challenges that may arise when comparing developmental myelination and remyelination due to injury. A few examples of these challenges include dysregulated levels of trophic support factors, inhibitors such as myelin debris might be present, injured axons and inflammation in the injury site (Plemel et al., 2017). In the context of MS, there are many factors that contribute to the failure of remyelination.

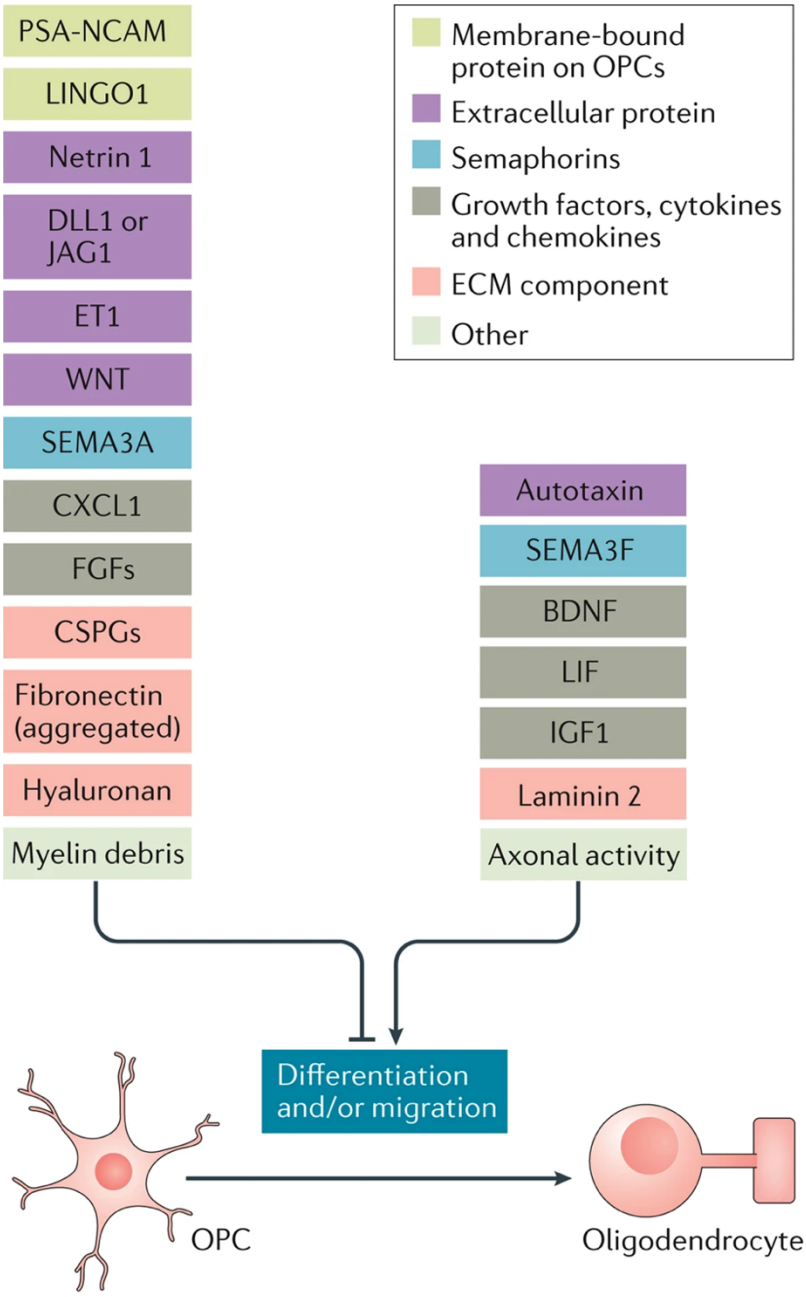


Figure 3: Regulation of oligodendrocyte precursor cell differentiation and remyelination. Many factors can promote or inhibit OPC differentiation towards myelinating-OLs. Factors such as myelin debris are seen in MS and specific to remyelination. Abbreviations: brain-derived neurotrophic factor (BDNF), chondroitin sulfate proteoglycans (CSPGs), delta-like 1 (DLL1), extracellular matrix (ECM), fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF1), jagged 1 (JAG1), leukemia inhibitory factor (LIF), polysialated neural cell adhesion molecule (PSA-NCAM), semaphorin 3 (SEMA3) (from Plemel et al., 2017).

1.2.2 Remyelination in Multiple Sclerosis

Degradation of demyelinated axons in MS contributes to the neurodegeneration that further exacerbates the disease course. Injury to myelin is observed in the white and gray matter in pwMS. Cortical lesions in the gray matter are correlated with cognitive impairment (Roosendaal et al., 2009). Demyelinated axons rearrange their sodium channels along the axon which increases the energy demand. Furthermore, OLs may have altered potassium clearance and nutritional support abilities which no longer meet the metabolic requirements of the injured axon (Lubetzki et al., 2020). Demyelination also predisposes unprotected axons to molecular cytotoxicity mechanisms and failure to derive neurotrophic support for myelinating OLs (Chari, 2007).

The remyelinating capacity for pwMS is highly variable. Remyelination capacity is reduced in MS due to a variety of factors such as signaling cues, insufficient levels of pro-regenerative factors and impaired OPC function. The presence of axonal inhibitors in lesion areas contribute to the unfavourable microenvironment for remyelination. OPC dysfunction can stem from inhibition of recruitment or inhibition of differentiation into mature OLs. Varying numbers of OPCs can be found in chronically demyelinated lesions. In general, age is inversely correlated to the number of OPCs recruited to lesions decreases (Wolswijk, 2002). OPCs are also limited in MS lesions due to their inability to differentiate. This block has been shown to be a key determinant in remyelination failure seen in chronic MS lesions (Kuhlmann et al., 2008). Chondroitin sulfate proteoglycans (CSPGs) are also known to inhibit remyelination by inhibiting OPC adhesion and differentiation in the lesion site (Lau et al., 2012). *Fluorosamine*, a CSPG synthesis inhibitor, promotes remyelination in animal models of demyelination in vivo (Keough

et al., 2016). Furthermore, *Clemastine*, an oral antihistamine approved for pwMS, also shows positive remyelination potential by increasing numbers of myelinating-OLs (Green et al., 2017).

Histologically, shadow plaques are an indicator of remyelination. The caveat of this assessment of remyelination is that these samples can only be obtained and evaluated post-mortem. Certain new imaging techniques are currently being developed to assess remyelinating capacity in vivo for pwMS. The combination of positron emission tomography with [^{11}C] PiB showed a significant correlation between individual remyelination potential and clinical disability (Bodini et al., 2016). Age and disease duration also affect the remyelinating capacity for pwMS. Increasing age, which is also correlated to disease progression, mitigates the remyelination potential in humans. There are a higher number of shadow plaques in pwMS under the age of 55 or within the first decade of the disease course (Frischer et al., 2015). A potential cause for the reduced remyelinating capacity is the inhibition of myelin debris removal from the injury site (Lampron et al., 2017; Plemel et al., 2017; Syed et al., 2008).

Table 1: Examples of some causes for remyelination failure in multiple sclerosis.

Examples of some causes for remyelination failure in MS	
Extrinsic inhibitors • CSPGs inhibit recruitment of OPCs by attenuating migration and impairing morphological differentiation • Notch receptor activation • Myelin debris in the lesion	*Keough et al., 2016; Lau et al., 2012; Plemel et al., 2013; Syed et al., 2008; Wang et al., 1998*
Aging • Reduces remyelination efficiency	*Shields et al., 1999; Sim et al., 2002*
Insufficient proregenerative factors	*Plemel et al., 2017*
Intrinsic capabilities of oligodendrocyte lineage cells • Differentiation block	*Kuhlmann et al., 2008*

1.2.3 Hypoxia, Remyelination, and Angiogenesis

There is a hypoxic signature demonstrated in the pathophysiology of MS. A review by Lassmann (2003) provides substantial evidence to support the argument that tissue with hypoxic

characteristics is a component of MS pathogenesis (Lassmann, 2003). Distal oligodendrogliopathy is a shared feature of white matter lesions in MS and acute hypoxic tissue damage. Type III MS lesions show hypoxic signature and lesions form in locations that are more susceptible to hypoxia (Brownell & Hughes, 1962). Reduced cerebral metabolism and oxygenation of tissue is also found diffusely throughout the CNS in MS (Sun et al., 1998; Yang & Dunn, 2015).

The hypoxia-inflammation cycle contributes to exacerbation of MS inflammation. It is cyclic because hypoxia can exacerbate inflammation and vice versa. Prolylhydroxylase (PHD) is responsible for breaking down hypoxia inducible factor 1 alpha (HIF-1) which is the master regulator during the hypoxia response. Hypoxia inhibits PHD, which in turn activates HIF-1. The downstream consequence is the activation of nuclear factor kappa B (NF-kB) which is involved in activating a proinflammatory response (Yang & Dunn, 2019). Concurrently, inflammation impairs vasoreactivity which allows increased leukocyte infiltration. The influx of immune cells causes an increase in oligodendrocyte and other cellular metabolic demands, which can exacerbate the hypoxic environment. OL's are highly susceptible to hypoxia in animal models of demyelination (Sosa & Smith, 2017). Treatment with oxygen improved viability of myelin-producing cells. Furthermore, impaired hypoxic signaling impairs remyelination and is involved in glial scar formation in animal models of ischemia (Wang et al., 2022).

During remyelination, angiogenesis occurs in order to maintain vascularity with new cells. Angiogenesis is a dynamic process of creating new blood vessels from existing vasculature. This process occurs in both physiological and pathophysiological condition. Typically, changes in cellular metabolism drive proportional changes in the local vasculature. For example, increased exercise will drive increased angiogenesis supplementing skeletal muscle

and cardiac tissue. There are two types of angiogenesis: sprouting and intussusceptive (Adair & Montani, 2010). Sprouting angiogenesis is the formation of new vessels from pre-existing ones. Typically, hypoxia initiates the sprouting angiogenic cascade via secretion of vascular endothelial growth factor A (VEGF-A). VEGF is also known to increase neuron resistance to injury and regulate OPC survival and differentiation (Girolamo et al., 2014). There are other angiogenic factors that also significantly contribute to vascular modelling such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and angiopoietins (Fallah et al., 2019). Intussusceptive angiogenesis is the splitting of one vessel into two. This type of angiogenesis predominantly creates capillaries where there are pre-existing capillary beds. Angiogenesis in MS is important to ensure that oxygen and nutrients are provided during myelin generation and to counteract the negative effects of hypoxia.

Promotion of angiogenesis in MS has been proposed to attenuate cerebral hypoperfusion (Abbasi-Kangevari et al., 2019). OPC transplant has been shown to promote angiogenesis through Wnt signaling in an animal model of stroke (Wang et al., 2022). Furthermore, several vascular growth factors, such as VEGF and EGF, can stimulate oligodendrocyte development (Baydyuk et al., 2020). Angiogenesis, hypoxia, and remyelination maintain complex interactions that can be altered in the context of a disease such as MS. Research targeting these factors may provide useful guidance in directing clinical interventions for pwMS.

1.3 Remote ischemic conditioning

1.3.1 History and Development

Remote ischemic conditioning (RIC) is an intervention where repeated periods of brief ischemia followed by reperfusion are applied to a remote limb, which confer global systemic protection. This has been traditionally used against hypoxic pathologies, such as stroke or organ

transplantation (Heusch et al., 2015). During ischemic injury, timely reperfusion is essential to diminish tissue necrosis. However, reperfusion is often considered a double-edged sword because it can also inflict further damage. The concept of RIC originated in 1986 when four cycles of ischemia/reperfusion were applied to the coronary artery of a canine (Murry et al., 1986). They showed that animals who received brief periods of direct ischemia to the heart had a 25% decrease in infarct size compared to controls when both groups were induced with myocardial infarctions. This concept was named ischemic preconditioning. However, direct ischemia of organs can be invasive and clinical translation is not always feasible, particularly in the CNS. In a canine model, brief ischemia to the circumflex branch conferred protection to the naïve myocardium from the subsequent induced coronary artery occlusion (Przyklenk et al., 1993). Following these two publications, the concept of *remote* ischemic conditioning gathered interest.

There are currently three temporal variants of RIC: preconditioning, perconditioning and postconditioning. Remote ischemic preconditioning (RIPreC) is used when the conditioning occurs prior to the injurious stimulus. Remote ischemic postconditioning (RIPostC) is used when the conditioning occurs after the stimulus. RIPreC and RIPostC are the more common variants of the intervention. The third variant is perconditioning (RIPerC), which occurs when the conditioning is occurring at the same time as the stimulus. Many studies have compared different time paradigms in animal models to evaluate the optimal duration of ischemia and reperfusion to produce beneficial effects (Zhou et al., 2018). RIC has been studied in a multitude of fields and there is no consensus on the optimal "dose-response" of the intervention. Based on feasibility and positive outcomes, the most supported conditioning paradigm is five minutes of ischemia followed by five minutes of reperfusion, repeated four times (Johnsen et al., 2016; Ripley et al.,

2021). Most of the ongoing basic science research with RIC stems from the stroke and cardiovascular fields. It has been shown that a variety of paradigms at all temporal variants (pre-, per- and post-) are effective in reducing infarct size in animal stroke models (Ripley et al., 2021).

Remote ischemic conditioning can also be used as a single or repeated "dose". Most of the current literature in the field focuses on evaluating a single application of RIC. The concept of repeated RIC has not been fully explored. One study looked at the effect of a single vs. repeated application of RIC in a rat model of myocardial infarct (Wei et al., 2011). The study concluded that a single application of RIC reduced infarct size, which had been previously shown in literature. Of interest, the repeated application of RIC further reduced the adverse long-term effects of myocardial left ventricular remodelling and improved the survival of the rats in a dose-dependent manner. This is one of the first publications to suggest that repeated RIC may have a "dose-dependent" effect. Furthermore, variations in the timing of the application of the intervention can yield significantly different results. Research in a mouse model of stroke evaluated postconditioning at different time points (Doeppner et al., 2018). Very delayed application of conditioning (five days post-injury) yielded a pleiotropic effect that showed sustained neurological recovery. On the contrary, early conditioning (< 24 hours post-injury) demonstrated transient neuroprotection. Altogether, there is strong support for RIC as an adjunct clinical treatment.

1.3.2 Proposed Mechanisms

The two mechanisms that have been primarily investigated regarding how a remote event in a limb can induce protection in systems such as the CNS are the humoral and neural hypotheses (Heusch et al., 2015). The humoral hypothesis proposes that conditioning causes an increase in

local protective factors, which is then conferred to remote organ systems through secretion into the bloodstream. The neural pathway proposes that remote conditioning activates local neural pathways, which subsequently activate remote pathways which may release local neuroprotective factors.

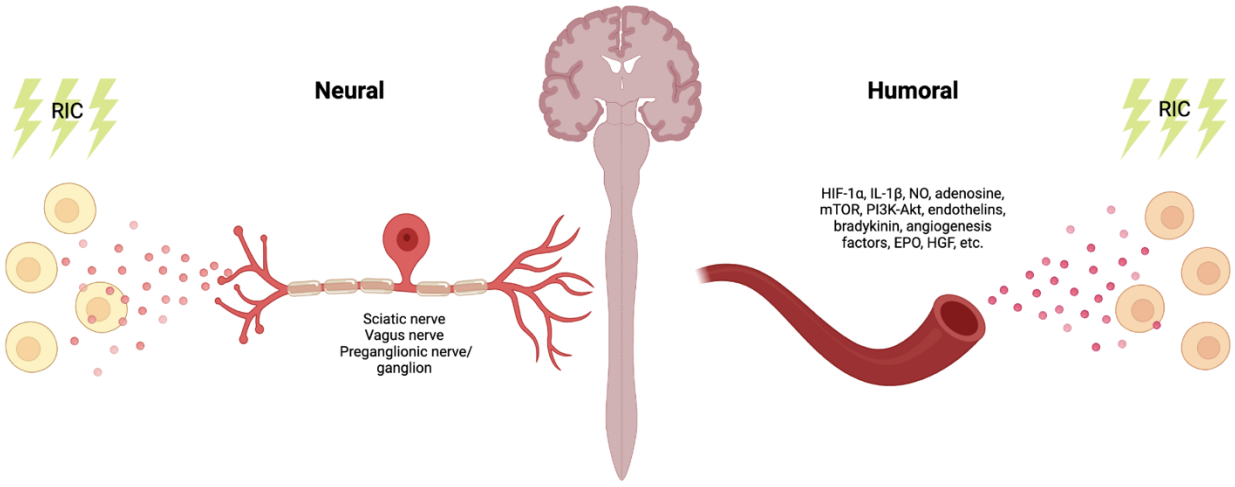


Figure 4: Neural and humoral pathway in remote ischemic conditioning. Abbreviations: hypoxia inducible factor-1 alpha (HIF-1α), interleukin-1 β, nitric oxide (NO), mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase-protein kinase B (PI3K-Akt), erythropoietin (EPO), hepatocyte growth factor (HGF). Created with Biorender.

Evidence for the humoral pathway has been demonstrated in cardioprotection studies. In this study, the researchers transferred blood samples from athletes that underwent RIC to mice that received induced ischemia-reperfusion injury (Jean-St-Michel et al., 2011). The mice that received RIC serum had significantly reduced infarct sizes when compared to animals that were perfused with control serum. Furthermore, occlusion of the femoral artery ablates the RIC-induced cardioprotection to the heart in a mouse model of myocardial infarct (Lim et al., 2010). However, the same authors also showed the converse argument, wherein femoral and sciatic nerve resection also ablated the RIC-induced cardioprotection in the same animal model. Further supporting the neural hypothesis, transection of the vagus nerve ablates the improved left ventricular function and reduced infarct size that was attributed to RIC (Donato et al., 2013).

It is likely that the neural and humoral RIC mechanisms both play a role in RIC. In a rodent model of myocardial infarct, it was determined that the activation of the vagus nerve through the neural pathway was necessary to release the RIC-associated protective factor (Pickard et al., 2016). The authors demonstrated that the resection of the vagus nerve did not induce the same cardioprotection as previously described. They also showed that only blocking the release of the humoral mediator, but allowing the vagus nerve to remain intact also did not induce the same level of cardioprotection. This concept was also supported in a clinical experiment involving patients with type 2 diabetes (Jensen et al., 2012). This group of researchers took dialysate from diabetic and non-diabetic patients after RIC. They transferred this dialysate into an animal model of ischemia-reperfusion injury. They showed that the cardioprotection was induced by the release of a cardioprotective factor into the humoral system, which subsequently activated a neural pathway. Taken together, this demonstrates the ways in which both the humoral and neural pathways are likely cooperative in the protection from RIC.

Another significant gap in knowledge regarding RIC concerns the primary drivers that cause these pleiotropic effects. A multitude of cytokines, proteins and other substances have been proposed as key molecules in the RIC pathway. One such study investigated 25 proposed cytokines and growth hormones in patients undergoing coronary revascularization and RIPreC as potential mediators of cardioprotection (Gedik et al., 2017). They concluded that only IL-1α fit their criteria as a potential mediator of RIPreC. HIF-1α and brain-derived neurotrophic factor (BDNF) have also been proposed as two significant mediators in protection induced by RIC in stroke research (Geng et al., 2021). Table 2 shows a sample summary of proposed RIC mediators based on the current literature. It is likely that many proteins, interleukins, cytokines, and molecules are involved in the mechanism underlying RIC.

Table 2: Examples of recent literature that proposes mediators of remote ischemic conditioning

Author(s), Year	Title	Paradigm	Proposed Mediator
Cellier et al., 2016	Remote Ischemic Conditioning Influences Mitochondrial Dynamics	5i/5r x 4	OPA1
Chen et al., 2020	Exosomes isolated from the plasma of remote ischemic conditioning rats improves cardiac function and angiogenesis after myocardial infarction through targeting Hsp70	2i/2r x 10	Hsp70, eNOS, HIF-1α, Ang-1, and VEGF
Doeppner et al., 2018	Very delayed RIPostC induces sustained neurological recovery by mechanisms involving enhanced angiogenesis and peripheral immunosuppression reversal	10i/10r x 3	Hsp70
England et al., 2017	RECAST (Remote Ischemic Conditioning After Stroke Trial): A Pilot Randomized Placebo Controlled Phase II Trial in Acute Ischemic Stroke	5i/5r x 4	Hsp27 and pHsp27
Gedik et al., 2017	Potential humoral mediators of remote ischemic preconditioning in patients undergoing surgical coronary revascularization	5i/5r x 3	IL-1α
Honda et al., 2019	Acute and chronic remote ischemic conditioning attenuates septic cardiomyopathy, improves cardiac output, protect systemic organs, and improves mortality in a lipopolysaccharide-induced sepsis model	5i/5r x 4	TNFα, IL-6, and IL-1β
Hausenloy et al., 2012	Underlying Remote Ischemic Conditioning in the Porcine Heart	5i/5r x 4	PI3K-Akt
Koike et al., 2020	Remote ischemic conditioning counteracts the intestinal damage of the necrotizing enterocolitis by improving intestinal microcirculation	5i/5r x 4	NO and H2S
Lassen et al., 2021	Cardioprotection by remote ischemic conditioning is transferable by plasma and mediated by extracellular vesicles	5i/5r x 4	miR-16-5p, miR-144-3p, and miR-451a
Li et al., 2022	Limb remote ischemic conditioning promotes neurogenesis after cerebral ischemia by modulating miR-449b/Notch1 pathway in mice	10i/10r x 3	Notch1 and miR-449b
Liu et al., 2020	Remote ischemic conditioning reduced cerebral ischemic injury by modulating inflammatory responses and ERK activity in type 2 diabetic mice	10i/10r x 3	IL-6 and pERK
Miyake et al., 2020	The effect of pre- and post-remote ischemic conditioning reduces the injury associated with intestinal ischemia/reperfusion	5i/5r x 4	IL-6
Rohailla et al., 2014	Acute, Delayed and Chronic Remote Ischemic Conditioning Is Associated with Downregulation of mTOR and Enhanced Autophagy Signaling	5i/5r x 4	AMPK, mTOR, LC3-I/II, and Atg5
Wang et al., 2021	Atg5 knockdown induces age-dependent cardiomyopathy which can be rescued by remote ischemic conditioning	5i/5r x 4	Atg5, mTOR, LC3-I/II, p62, and beclin
Wei et al., 2012	The Chronic Protective Effects of Limb Remote Preconditioning and the Underlying Mechanisms Involved in Inflammatory Factors in Rat Stroke	15i/15r x 3	iNOS, galectin-9, and Tim-3
Yamaguchi et al., 2015	Repeated RIC attenuates left ventricular remodeling via exosome-mediated intercellular communication on chronic heart failure after myocardial infarction	5i/5r x 5	miR-29a and IGF-1
Yang et al., 2018	Hypoxia Inducible Factor 1α Plays a Key Role in Remote Ischemic Preconditioning Against Stroke by Modulating Inflammatory Responses in Rats	10i/10r x 3	HIF-1α, IL-4, and IL-10

As previously described, HIF-1α is a key mediator of hypoxia signaling and thus a proposed mediator of the mechanism underlying remote ischemic conditioning. An early study revealed that HIF-1 was a central mediator of cardioprotection in ischemic preconditioning studies (Eckle et al., 2008). They initially showed that ischemic preconditioning induced cardioprotection in mice. The researchers then applied a HIF activator or a small interfering RNA of PHD-2 (HIF-1 repressor) with ischemic preconditioning. The study concluded that HIF-1 is a central mediator of ischemic preconditioning-induced cardioprotection through signaling with the A2B adenosine myocyte receptor. This line of research expanded to test whether HIF-1 also induced protective effects with remote conditioning. One study tested whether the effects of RIC could be attenuated using a HIF-1 transcriptional inhibitor, *Acriflavine* (Cai et al., 2013). This study showed that transcriptional activation of HIF-1 is required to induce the *IL10* gene in myocytes to demonstrate RIC-induced cardioprotection. A similar study also showed that HIF-1 transcriptional inhibitor acriflavine hydrochloride also abolished the protective effects of RIC in a rat stroke model (Yang et al., 2018). In addition, they used a HIF activator dimethyloxaloylglycine and showed that it had the same protective effects as RIC. In a pilot clinical study, RIC induced increased levels of HIF-1α and procaspase-3 levels in serum (Albrecht et al., 2013).

Many signaling pathways have also been involved in elucidating the mechanism underlying RIC. The PI3K-Akt pathway is a major signaling pathway for many processes such as cell growth, cell cycling and proliferation. Ischemic preconditioning increased levels of Akt phosphorylation and reduced myocardial infarct in a mouse model (Rossello et al., 2018). The study also showed that blocking the PI3K-Akt pathway attenuated the reduction of infarct size and Akt phosphorylation induced by ischemic preconditioning. Downregulation of mTOR

pathways and upregulation of autophagy pathways have also been associated with RIC (Rohailla et al., 2014). Increased mTOR signaling is often described in cancer and tumour growth and regulates gene transcription, protein synthesis and protein metabolism (Saxton & Sabatini, 2018). In simple terms, autophagy is the process of recycling old and/or injured cells (Misrielal et al., 2020). However, several other pathways have been associated with RIC such as oxidative stress, inflammation, angiogenesis, posttranscriptional modifications (Chen et al., 2020; Kohns et al., 2019; Monteiro et al., 2021). Furthermore, RIC not only directly affects hypoxia pathways, but also promotes the release of pro-angiogenic factors. For example, RIC induces the upregulation of VEGF and VEGF-dependent signaling in animal models (Kambakamba et al., 2018). VEGF is one of the key regulators of angiogenesis and is involved in neuroprotection.

1.3.3 Clinical success using remote ischemic conditioning

Much of clinical RIC research has been focused on the cardiovascular field. RIPerC has been shown to be an effective adjunct therapy for patients undergoing valve replacement surgery (Hu et al., 2016). The researchers found that patients in the RIPerC group had reduced drainage and myocardial damage when compared to the control surgery group. Another clinical trial used RIPostC on patients with ST-segment elevation myocardial infarction (White et al., 2015). The study concluded that patients in the RIPostC group had reduced infarction size and myocardial edema. A trial involving patients with small-vessel ischemic disease showed that one year of daily, bilateral upper limb RIC improved middle cerebral artery hemodynamics and reduced white matter lesion volumes (Mi et al., 2016). Another chronic condition trial focused on patients with intracranial artery stenosis (Meng et al., 2012). They showed that 300 days of daily, bilateral limb RIC reduced stroke recurrence in these patients. A recent, large-scale RCT evaluated the use of remote ischemic conditioning on neurological function in patients with acute

moderate ischemic stroke (Chen et al., 2022). The researchers split 1893 patients into two groups; one group received five cycles of five minutes of ischemia and five minutes of reperfusion daily for 10-14 days following symptom onset in adjunct to their respective treatments. The other group received no adjunct and acted as a control group. The results showed that the RIC group had a statistically significantly increased likelihood of excellent neurologic function at 90 days post-stroke. These recent successes suggest that RIC can be relatively easily integrated into a patients adjunct care and that this intervention has clinical efficacy.

1.3.4 Remote ischemic conditioning and multiple sclerosis

There are multiple theoretical avenues for which RIC could play a role in MS research (Camara-Lemarroy et al., 2018). One idea suggests that MS pathophysiology contains an ischemia/hypoxia component, which may be altered with this ischemia-targeted intervention, as previously described. Chronic hypoxia and tissue hypoperfusion have been described in pwMS (D'haeseleer et al., 2015). As aforementioned, OPCs are also very susceptible to hypoxic conditions. EAE mice that are conditioned to hypoxia show decreased levels of CD4+ T cells and delayed EAE onset (Esen et al., 2016). RIC may provide an alternative method of conditioning that also targets attenuating the consequences of hypoxia in MS pathophysiology.

Previous literature has shown that RIC can reduce injury in other models of white matter damage. In an animal model of vascular cognitive impairment and dementias, researchers explored the use of RIC for one and four months (Khan et al., 2018). This study concluded that RIC induced increased angiogenesis and preservation of white matter. Another study investigated the neuroprotective capabilities of RIC in an optic nerve crush model (Liu et al., 2013). The researchers found that postconditioning demonstrated survival and protection of the

white matter tract and retinal ganglion cells. Clinically, RIC improved white matter hyperintensities in elderly patients with intracranial atherosclerotic stenosis (Zhou et al., 2019).

RIC is a broad intervention that affects a multitude of signaling pathways that could also be dysregulated in MS. Previous studies have described RIC modulating antioxidant and anti-inflammatory pathways (Liu et al., 2016). Ischemic preconditioning shifts CNS glial cells towards a phenotype that generates trophic and cellular support (McDonough & Weinstein, 2016). In addition, oxygen therapy has shown restoration of function in the EAE model (Davies et al., 2013). In efforts to characterize molecular signatures of NAWM in pwMS, research has shown that there is an upregulation of genes that have been associated with RIC such as HIF-1α and PI3K/Akt signaling (Graumann et al., 2003).

Targets that are altered due to RIC may play a role in white matter protection and repair in MS. Neuroprotective factors such as VEGF-A and BDNF are upregulated due to application of RIC (Guo et al., 2019; Xu et al., 2021). Researchers have proposed that BDNF plays a role in modulating neuroinflammation and inducing neuroprotection in pwMS (Nociti & Romozzi, 2023). VEGF has been proposed as a factor that enhances neuron resistance to injury and OPC migration to lesions in pwMS (Girolamo et al., 2014). HGF-cMet signaling has also been shown to increase due to conditioning in animal models. In the context of MS, HGF induces recovery in animal models by promotion of mature oligodendrocyte development and remyelination (Bai et al., 2012). Based on current literature, it is unclear whether RIC may be a suitable intervention for MS intervention. However, RIC is a feasible and relatively accessible intervention that can be hypothesized to provide benefits in the context of MS. The link between RIC and MS pathophysiology requires further exploration to support any definitive conclusions.

1.4 Hypothesis and experimental aims

This thesis investigates the effects of remote ischemic conditioning in the CNS and within the context of an animal model of MS. In order to test develop this investigation, we address three specific aims:

Aim 1: Develop and animal model of repeated remote ischemic conditioning.

Aim 2: Investigate the effects of repeated remote ischemic conditioning in the spinal cord.

Aim 3: Evaluate changes in the demyelinated lesion environment due to repeated remote ischemic conditioning.

We hypothesized that we would see broad, systemic changes with repeated remote ischemic conditioning that can be conferred to the CNS. We expect to see upregulation of pathways and markers that are associated with protection against white matter damage observed in pwMS. Lastly, we expect to see changes in the lesion environment in an animal model of MS that are neuroprotective.

Chapter 2: Methods

2.1 Animal care

All animals were conducted with ethics approval from the Animal Care Committee at the University of Calgary under regulations of the Canadian Council of Animal Care. All the mice used were C57BL/6 mice from 6-8 weeks of age purchased from Charles River Laboratories (Québec, Canada). Mice were maintained on a 12-hour light/dark cycle with water and food provided ad libitum.

2.2 Spinal cord harvest and sectioning

Mice were sacrificed through overdose of ketamine/xylazine, followed by intracardiac perfusion of ~15 mL of PBS, followed by ~15 mL of 4% PFA to fix the tissue. Spinal cords were then dissected from the dorsal side of the mouse. The cervical spinal cord to the lower thoracic region was taken and placed into microtubes containing 1 mL of 4% PFA and were stored at 4ºC for 24 hours. After this fixation, the spinal cords were transferred to a 30% sucrose solution for 72 hours. Spinal cords were then placed in Frozen Section Compound 22 Clear (Leica, 3801480) and froze over dry ice and 2-methylbutane (Sigma, 78-78-4). The cords were then cut in 20 µm sections on a cryostat (ThermoFisher), and sections were collected on Superfrost® Plus microscope slides (VWR, 48311-703) and stored at -20ºC for subsequent analysis.

2.3 Remote ischemic conditioning paradigm

Equal numbers of mice were randomly assigned to the following groups: Single RIC, repeated RIC or sham. Mice that underwent conditioning were anesthetized using 100mg/kg of ketamine-xylazine cocktail. RIC mice were laid flat on a heating pad (Kent Scientific) and both hindlimbs were attached to custom sphygmomanometer cuffs. The sphygmomanometers used

were Gold Series DS66 Trigger Aneroid Gauges (Cardinal Health) attached to Durashock inflation bulbs (Cardinal Health). The cuffs used for the mouse hindlimbs were custom designed UDC 1.0cm x 4.0cm durable polyurethane digit cuffs (Hokanson Vascular). The sphygmomanometers were inflated to a maximum pressure of 300mmHg for five minutes of limb ischemia. The pressure was then released to 0mmHg for five minutes of reperfusion. This cycle occurred four times, for a total of 40 minutes per mouse. For the single RIC group, conditioning was performed once and then immediately sacrificed. For the repeated RIC group, mice were conditioned at approximately the same time every day for 14 consecutive days. After the 14^{th} cycle, the mice were immediately sacrificed. The matched sham groups received anesthesia and were attached to the sphygmomanometers for 40 minutes, but the cuffs were never inflated. In the injury model, animals were conditioned after the induction of the injury. Thus, these animals underwent remote ischemic postconditioning (RIPostC).

2.4 Lysolecithin (lysophosphatidylcholine)-induced demyelination

Mice were anaesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally. Skin overlying surgical site was shaved and disinfected with 70% ethanol and iodine. Ophthalmic gel was applied to both eyes to prevent drying, and buprenorphine (0.05 mg/kg) was injected subcutaneously immediately prior to surgery and 12 hours post-surgery as an analgesic. Animals were positioned on a stereotaxic frame and a midline incision approximately 5 cm long was made between the shoulder blades using a #15 scalpel blade. A retractor was used to separate the muscle and adipose tissue to expose the spinal column. The prominent T2 vertebra was used as a landmark to find the T3–T4 intervertebral space. Tissue in the T3–T4 gap was then blunt dissected apart using forceps and spring scissors, and the dura was

removed using a 30-gauge metal needle. Using a 32-gauge needle attached to a 10 μL Hamilton syringe, 0.5 μL of 1% lysolecithin/lysophosphatidylcholine (LPC) (Sigma-Aldrich, L1381) was injected into the ventral column of the spinal cord at a rate of 0.25 μL/min for 2 min. The needle was left in place for 2 min following the injection to avoid back flow, followed by suturing of the muscle and skin. Mice were then placed in a thermally controlled environment for recovery and assessed daily for open sutures or signs of discomfort.

2.5 Tissue multiplex

To study changes in angiogenesis-related targets, we used the Mouse Angiogenesis 25-plex (MAGP-25-106, Eve Technologies, Calgary, Canada). This array tests for levels of amphiregulin, angiopoietin-2, epidermal growth factor, endoglin, endothelin-1, fas ligand, fibrocyte growth factor 2, follistatin, granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor, interleukin (IL)-1β, IL-6, IL-17, KC, leptin, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, placenta growth factor 2, prolactin, activin receptor-like kinase 1, stromal cell-derived factor 1, tumor necrosis factor 1, vascular endothelial growth factor (VEGF)-A, VEGF-D, and VEGF-D.

To study an array of cytokines and chemokines, we used the Mouse Cytokine/Chemokine 45-Plex Discovery Assay Array (MD45, Eve Technologies, Calgary, Canada). This array tests for levels of eotaxin, erythropoietin, 6Ckine, fractalkine, G-CSF, GM-CSF, IFNβ1, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-16, IL-17, IL-20, IP-10, KC, LIF, LIX, MCP-1, MCP-5, M-CSF, MDC, MIG, MIP-1α, MIP-1β, MIP-2, MIP-3α, MIP-3β, RANTES, TARC, TIMP-1, TNFα, and VEGF-A. We also used two smaller arrays. The first was the TGFβ 3-Plex Discovery Assay Multi Species Array (TGFβ1-3,

Eve Technologies, Calgary, Canada). This array tests for levels of TGFβ1, TGFβ2, and TGFβ3. The second array was the Mouse IGF-1 Single Plex (MIGF1-01-101, Eve Technologies, Calgary, Canada). This array tests for levels of IGF-1. Details of the multiplex method and limits of detection of this assay are available online (https://www.evetechnologies.com).

2.6 Bulk RNA sequencing

Since RIC involves multiple pathways, we used bulk RNA sequencing to evaluate any transcriptomic changes occurred in the CNS with repeated RIC. 24 animals were split into three groups (n=8 per group): sham, 1 day of 5i/5r RIC (1d) and 14 days of 5i/5r repeated RIC (14d). No animals received other treatment (ex: LPC injury). Upon final conditioning treatment, animals were sacrificed and harvested as previously described in Methods 2.2. Instead of paraformaldehyde fixation, the freshly harvested spinal cord sections were flash frozen in liquid nitrogen and stored in -80ºC until use. RNA isolation from the spinal cords was performed using a QIAGEN RNeasy Mini Kit kit and protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-kits?catno=74104). Bulk RNA sequencing was completed in collaboration with Dr. Paul Gordon and the University of Calgary's Centre for Health Genomics and Informatics (https://cumming.ucalgary.ca/research/cat/health-genomics/home). The samples were processed using the New England Biolab's NEB Ultra II Directional RNA library preparation and rRNA depletion v2 kit (https://international.neb.com/protocols/2017/02/07/protocol-for-use-with-nebnext-rrna-depletion-kit-human-mouse-rat-neb-e6310-and-nebnext-ultra-ii-directional-rna-library-prep-kit-for-illumina-neb-e7760-e7765). The samples were then sequenced on a NovaSeq6000 100 cycle S1 flow cell with paired end, 2x51 base pairs.

2.7 Tandem mass tag labelling proteomics

Tissue was lysed in a buffer composed of 1% SDS, 200 mM HEPES (pH 8.0), 100 mM ammonium bicarbonate, 10 mM EDTA and protease inhibitor cOmplete tablets (Roche, 4693159001). Disulfide bonds of 100 μg of total protein were reduced with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (Thermo Fisher Scientific) at 55°C for 1 h. The proteins were then alkylated by incubation with 15 mM iodoacetamide (VWR) for 25 min in the dark at room temperature. Proteins were precipitated out of solution by adding 600 μL of ice-cold acetone and incubated at -20°C overnight. Samples were centrifuged at 8,000 g for 10 min before resuspension in 100 μL of 50 mM triethyl ammonium bicarbonate. Proteins were then trypsinized (Thermo Fisher Scientific) overnight at a 1:10 enzyme-to-substrate ratio. For TMT 6-plex labeling, 0.8 mg of TMT reagent (Thermo Fisher Scientific) was resuspended in 41 μL of acetonitrile (ACN), samples were spun down quickly at 2,000 rpm (380g) for 10 s, and samples were incubated at room temperature for 1 h. The labelling reaction was quenched by adding 8 μL of 5% hydroxylamine and incubated for 15 min at 25°C. Peptides with different labels were combined before 100% formic acid was added to each sample to reach a volumetric concentration of 1% formic acid. Samples were spun at 5,000 rpm (2350 g) for 10 min and then desalted using Sep-Pak C18 columns (Waters, 130 mg WAT023501). Sep-Pak columns were conditioned with 1 × 3 mL 90% methanol/0.1% TFA, 1 × 2 mL 0.1% formic acid. Each sample was loaded onto a column and washed with 1 × 3 mL 0.1% TFA/5% methanol. Peptides were eluted off the column with 1 × 1 mL 50% ACN/0.1% formic acid and lyophilized. Peptides were resuspended in 1% formic acid and a BCA assay (Thermo Fisher Scientific) was used to determine the concentration of peptide in each sample. Samples were dried down and stored at −80°C.

2.8 High performance liquid chromatography and mass spectrometry

All liquid chromatography and mass spectrometry experiments were carried out by the Southern Alberta Mass Spectrometry (SAMS) core facility at the University of Calgary, Canada. In detail, analysis was performed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) operated with Xcalibur (version 4.0.21.10) and coupled to a Thermo Scientific Easy-nLC (nanoflow Liquid Chromatography) 1200 system. Tryptic peptides (2 µg) were loaded onto a C18 trap (75 µm x 2 cm; Acclaim PepMap 100, P/N 164946; Thermo Fisher Scientific) at a flow rate of 2 µL/min of solvent A (0.1% formic acid and 3% acetonitrile in LC-mass spectrometry grade water). Peptides were eluted using a 120 min gradient from 5 to 40% (5% to 28% in 105 min followed by an increase to 40% B in 15 min) of solvent B (0.1% formic acid in 80% LC- mass spectrometry grade acetonitrile) at a flow rate of 0.3 µL/min and separated on a C18 analytical column (75 um x 50 cm; PepMap RSLC C18; P/N ES803; Thermo Fisher Scientific). Peptides were then electrosprayed using 2.3 kV voltage into the ion transfer tube (300°C) of the Orbitrap Lumos operating in positive mode. The Orbitrap first performed a full mass spectrometry scan at a resolution of 120,000 FWHM to detect the precursor ion having a mass-to-charge ratio (m/z) between 375 and 1575 and a +2 to +4 charge. The Orbitrap AGC (Auto Gain Control) and the maximum injection time were set at 4e5 and 50 ms, respectively. The Orbitrap was operated using the top speed mode with a 3 sec cycle time for precursor selection. The most intense precursor ions presenting a peptidic isotopic profile and having an intensity threshold of at least 2e4 were isolated using the quadrupole (isolation window of m/z 0.7) and fragmented with HCD (38% collision energy) in the ion routing Multipole. The fragment ions (MS2) were analyzed in the Orbitrap at a resolution of 15,000. The AGC, the maximum injection

time and the first mass were set at 1e5, 105 ms and 100, respectively. Dynamic exclusion was enabled for 45 sec to avoid of the acquisition of same precursor ion having a similar m/z (plus or minus 10 ppm).

2.9 Bioinformatics and data analysis

Spectral data were matched to peptide sequences in the murine UniProt protein database using the Andromeda algorithm (Cox et al., 2011) as implemented in the MaxQuant (Cox & Mann, 2008) software package v.1.6.0.1, at a peptide-spectrum match false discovery rate (FDR) of < 0.01. Search parameters included a mass tolerance of 20 p.p.m. for the parent ion, 0.5 Da for the fragment ion, carbamidomethylation of cysteine residues (+57.021464 Da), variable N-terminal modification by acetylation (+42.010565 Da), and variable methionine oxidation (+15.994915 Da). TMT 6-plex labels 126 to 131 were defined as labels for relative quantification. The cleavage site specificity was set to Trypsin/P (search for free N-terminus and for only lysines), with up to two missed cleavages allowed. Next, quantified proteins were filtered using the Perseus software (Tyanova et al., 2016). Proteins that have more than two replicates with zero values in more than one group were removed. Filtered proteins were normalized using the CycLoess approach via NormalizerDE package using the R language (v3.6.0). An average of the normalized results for each group was calculated and followed by the ratio of each group comparison. The ratios were log(2) transformed and the significant outlier cut-off values were determined after log(2) transformation by boxplot-and-whiskers analysis using the BoxPlotR tool (Spitzer et al., 2014).

2.10 Reactome pathway analysis

To identify protein-protein interaction, the STRING (Search Tool for the Retrieval of Interacting Genes) database was used to illustrate interconnectivity among proteins. Protein interaction relationship is encoded into networks in the STRING v11 database (https://string-db.org). Our data was analyzed using the *Mus musculus* as our model organism at a false discovery rate of 5%. Proteins belonging to specific pathways were selected and their ratios were plotted as heatmaps.

2.11 Immunofluorescent staining

Microscope slides with mouse tissue samples were removed from -20ºC storage and left at RT to air dry for 30 min. For MBP staining, slides underwent delipidating with successive one-minute washes with 50%, 70%, 90%, 95%, 100%, 95%, 90%, 70%, and 50% ethanol. Horse blocking solution (PBS, 10% horse serum, 1% BSA, 0.1% cold fish stain gelatin, 0.1% Triton X-100, 0.05% Tween-20) was used to block all samples for one hour at RT. After blocking, slides were incubated with primary antibodies in antibody dilution buffer (PBS, 1% BSA, 0.1% cold fish stain gelation, 0.1% Triton X-100) overnight at 4ºC. Following this, slides were washed three times using a 0.05% Tween-20 in PBS solution for five minutes, then incubated with secondary antibodies at RT for one hour, protected from light. Slides were then washed three times with 0.05% Tween-20 in PBS for five minutes and coverslips were mounted with Fluoromount-G solution (SouthernBiotech, 0100-01).

2.12 Eriochrome cyanine staining

Briefly, spinal cords on microscope slides were removed from -20ºC storage and left to air dry at RT for 30 minutes. The slides were then subjected to citrisolv (Fischer Scientific, 22-143975), isopropanol (Sigma, 279544), 100% ethanol, 95% ethanol, 90% ethanol, 70% ethanol, and 50% ethanol in succession for one minute each. Slides were then washed in distilled water for one minute, followed by 15-minute treatment with an erichrome cyanine solution (10% FeCl3) and an immediate treatment with 0.5% NH4OH for 5 seconds preceding another one-minute wash with distilled water. Finally, samples were dehydrated with successive treatments of 50%, 70%, 90%, 95%, and 100% ethanol each for one minute, followed by two-minute washes in isopropanol and four minutes in citrisolv. Coverslips were then mounted on slides with Acrytol Mounting 36 Medium (Electron Microscopy Science, 13518) and left to dry at RT for 30 minutes. Brightfield images were acquired at 20x 0.75 NA air objective using the Olympus VS110 Slidescanner.

2.13 Confocal microscopy

Images were taken on the Leica TCS Sp8 laser confocal microscope using the 10x air objective and 25x water objective. 405nm, 488nm, 547nm and 647nm lasers were used to excite fluorophores with fluorophores with detection by two low dark current Hamamatsu PMT detectors and two high sensitivity hybrid detectors. Images were acquired in 8-bits in a z-stack bidirectionally, with 2-times line average and at 0.57mm optical sections. Laser levels were maintained across all sections of common stains. Images were blinded and analysed using ImageJ.

2.14 EF5 assay

To test the safety and development of ischemia in the limb, we used an EF5 hypoxia assay. Female C57Bl/6 mice were separated into two RIC groups: five minutes ischemia followed by five minutes of reperfusion, four times (5i/5r x4) or 40 minutes of ischemia. The EF5 hypoxia detection kit (Sigma-Aldrich) reagents were prepared as described in the manual. All animals received a tail vein injection of 1/100 (g to mL) of the animal weight (g) of 10mM (mL) EF5. For example, a 25-gram mouse would receive 0.25mL of 10mM EF5. The injection was done 90 minutes prior to their conditioning. Conditioning for the 5i/5r x4 group was performed as previously described in Methods 2.3. Animals in the second group underwent 40 uninterrupted minutes with cuffs inflated to 300mmHg to cause intentional hypoxia. In both groups, immediately following the conditioning paradigm, the mice were sacrificed as previously described in Methods 2.2. For tissue harvest, the anterior biceps femoris from the hindlimb was isolated and removed. The tissue was placed in Frozen Section Compound 22 Clear (Leica, 3801480) and froze over dry ice and 2-methylbutane (Sigma, 78-78-4). The muscle was then cut in 10 μm sections on a cryostat (ThermoFisher), and sections were collected on Superfrost® Plus microscope slides (VWR, 48311-703). As described in the assay protocol, the slides were fixed with 4% paraformaldehyde. Following fixation, blocking solution was applied and the slides were left overnight in 4ºC. The general EF5 staining procedure was followed and slides were mounted to be visualized on the Leica SP8 (Methods 2.11).

2.15 Statistical analysis

All statistical analyses were completed using Prism 9. P-value < 0.05 is flagged with one star (*), p-value < 0.01 is flagged with two stars (**) and p-value < 0.001 is flagged with three stars (***).

Chapter 3: Results

3.1 Development of the animal model of remote ischemic conditioning

Review of current RIC literature did not elucidate a specific temporal paradigm that is standard in the field. One study compared differences in multiple variations of RIC in the context of rodent myocardial infarct (Johnsen et al., 2016). Figure 5 shows a summary of the experimental design. The study demonstrated that four to six cycles of ischemia/reperfusion yielded the most robust cardioprotection when compared to other groups. There was no further cardioprotection demonstrated after eight cycles. Two or five minutes of ischemia demonstrated the same levels of protection. Furthermore, 10 minutes of ischemia attenuated the protection. Based on these findings and other literature (Sandhu et al., 1997; Sogorski et al., 2021), we determined that the RIC paradigm for our experiments would follow four cycles of bilateral five minutes of ischemia followed by five minutes of reperfusion on the hindlimbs. Furthermore, this is like what has been done in human studies and could thus help with the future translation of this project (Landman et al., 2019; Zhao et al., 2018). Figure 6 shows a visual set up of the RIC experiments. Mice were anesthetized during the entire intervention.

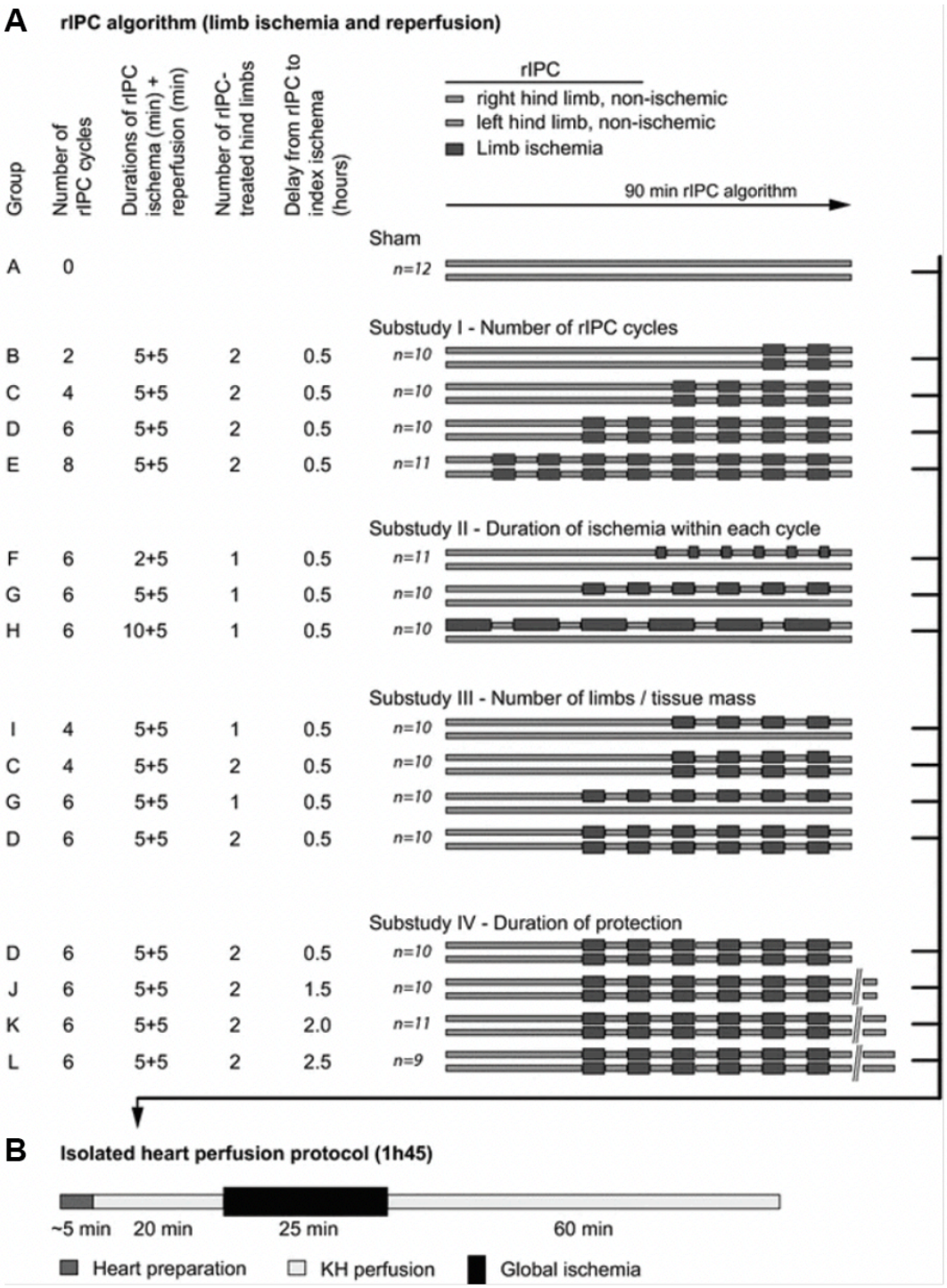


Figure 5: Study design of variants of remote ischemic conditioning in a mouse model of myocardial infarct. Remote ischemic preconditioning protocols (A) and in vitro heart perfusion protocol (B). The delay from remote ischemic preconditioning to index ischemia reflects the time from completion of the remote ischemic preconditioning protocol to ischemia, including the duration of the operative procedure and the isolated heart stabilization period (from Johnsen et al., 2016).

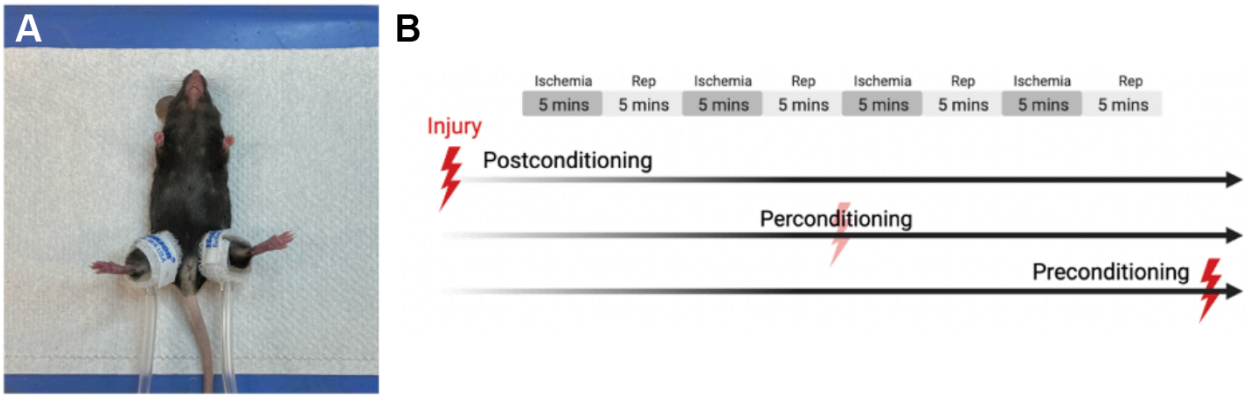


Figure 6: Experimental set up of remote ischemic conditioning. (A) Image of anesthetized mouse with bilateral hind limbs attached to sphygmomanometer (not shown). (B) Temporal outline of postconditioning, perconditioning, and preconditioning.

3.2 Evaluating the feasibility and safety of remote ischemic conditioning

In order to ensure that long-lasting hypoxia was not induced from the experiemental paradigm, a proof-of-concept EF5 hypoxia experiment was designed. The EF5 assay is marketed as a qualitative, not quantitative, assay. The results of the assay are shown in Figure 7. Two mice underwent RIC with a 5i/5r x4 protocol while two other mice underwent 40 minutes of consecutive ischemia. Representative images were taken from each group. Animals that underwent the 5i/5r x4 RIC protocol did not exhibit apparent EF5 staining in the muscle tissue. Animals that underwent 40 minutes of uninterrupted ischemia to the hindlimb demonstrated positive EF5 staining in the leg tissue.

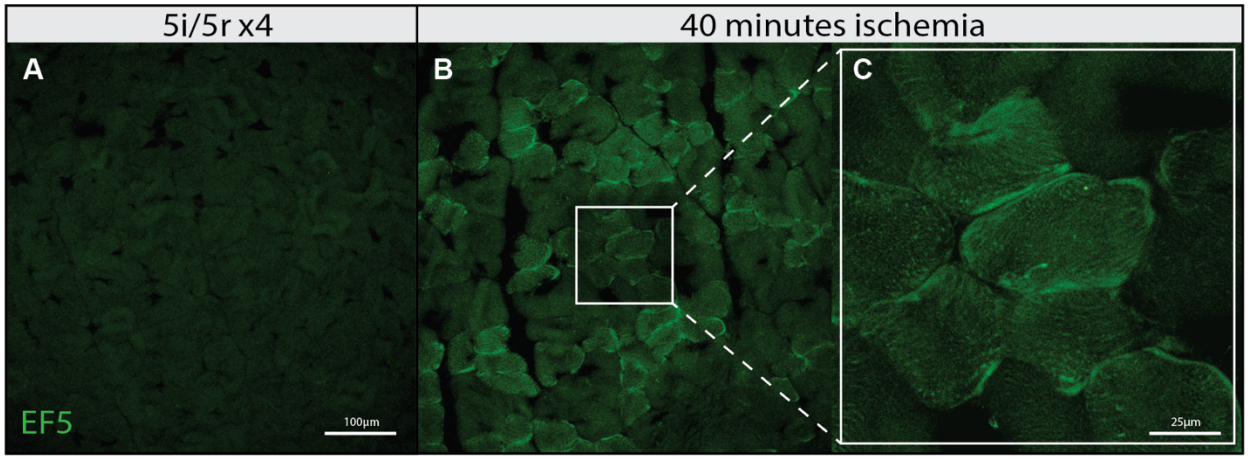


Figure 7: Hypoxia signature staining in mouse bicep femoris. (A) EF5 staining is shown in green for animals that received four cycles of five minutes of ischemia and five minutes of reperfusion (5i/5r x4) once (n=2). (B, C) EF5 staining is shown in green for animals that received 40 minutes of consecutive ischemia (n=2). Images were taken with 25x (A, B) and 40x (C) lens. Scale bars represent 100mm (A, B) and 25mm (C).

To further examine the development of the repeated RIC model, an observational experiment was performed. Physiological changes were recorded as shown in Figure 8. The baseline temperature of the hind paw of a mouse was typically 24±1°C. Upon inflation of the cuffs to 300mmHg, the temperature of the hind paw drops to 21±1°C. At baseline, the hind paw of the mouse is a pale pink color. When the cuffs are inflated, the color of the hind paw transitions into a paling gray color. During reperfusion (0 mmHg), the paw regains a pink color very quickly which transiently dulls to a faded pink throughout the five minutes. These characteristics held true across all mice that underwent the 5i/5r x4 cycle of RIC.

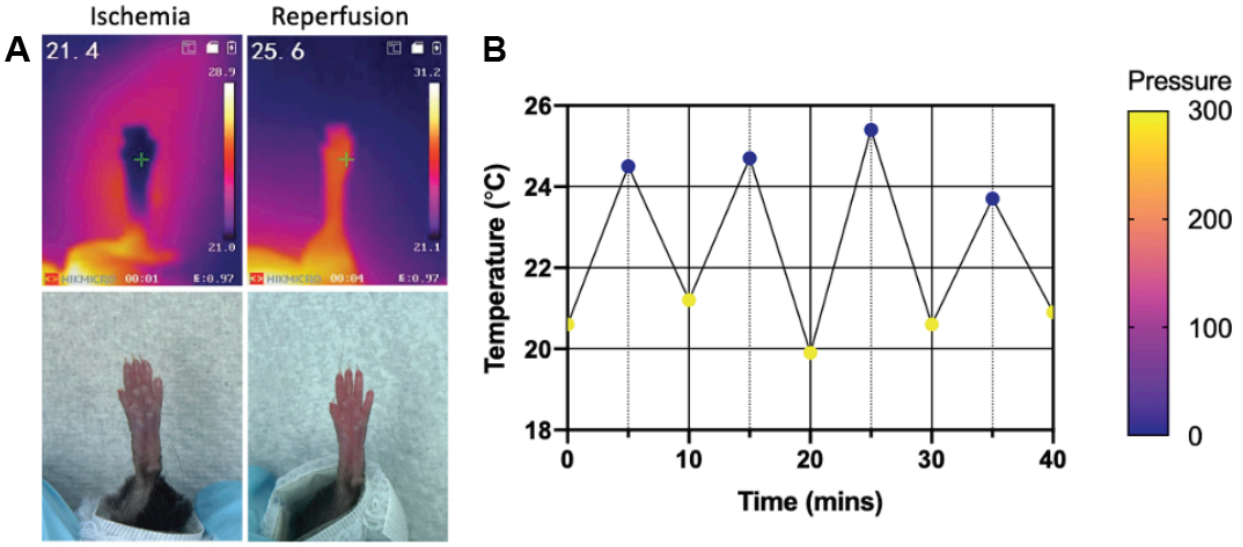


Figure 8: Observable changes to mice undergoing remote ischemic conditioning. (A) Infrared images were taken using a HIKMICRO EL1 Compact Thermal Imaging Camera. Images were taken using a dual 12MP camera at f/1.6 aperture. (B) Temperature and pressure changes were tracked and compiled using Prism 9.

3.3 Changes in systemic levels of BDNF and HGF with acute and repeated remote ischemic conditioning

Based on RIC literature, two targets were chosen as validation markers for the model. BDNF and HGF have been described as potential mediators of the RIC signalling pathway and have demonstrated neuroprotective effects experimentally (Desole et al., 2021; Ramagiri & Taliyan, 2017). Animals were split into three groups: sham conditioning, acute (1d) conditioning and repeated (14d) conditioning. Six mice were used in each of the three groups. Serum, liver homogenate and kidney homogenate were measured across groups for levels of HGF. Serum level of HGF measured: sham 2556.17 ± 1141.81pg/mL, 1d 5099.67 ± 1013.86pg/mL, and 14d 7746.83 ± 1221.80pg/mL. Statistical significance was revealed in comparing serum HGF sham vs. 14d ($p=0.0005$). HGF in kidney homogenates measured: sham 5216.33 ± 920.38pg/mL, 1d 7414.83 ± 835.76pg/mL, and 14d 7860.83 ± 1063.57pg/mL. Statistical significance was revealed in comparing kidney HGF sham vs. 14d ($p=0.0047$). HGF in liver homogenates measured: sham

5689.50 ± 448.30pg/mL, 1d 7535.50 ± 1352.50pg/mL, and 14d 9322.50 ± 1060.09pg/mL. Statistical significance was revealed in comparing liver HGF sham vs. 14d (p=0.0024).

Serum, liver homogenate and tissue homogenate were measured across groups for levels of BDNF. Serum level of BDNF measured: sham 99.00 ± 56.63pg/mL, 1d 348.80 ± 40.72pg/mL, and 14d 385.00 ± 95.69pg/mL. Statistical significance was found in comparing serum BDNF sham vs. 1d (p=0.0486) and comparing serum BDNF sham vs. 14d (p=0.0112). BDNF in kidney homogenates measured: sham 253.40 ± 132.56pg/mL, 1d 419.80 ± 77.91pg/mL, and 14d 339.40 ± 137.08pg/mL. No statistical significance was found in comparing levels of BDNF in the kidney homogenates across groups. BDNF in liver homogenates measured: sham 75.33 ± 35.50pg/mL, 1d 431.00 ± 205.35pg/mL, and 14d 155.17 ± 36.12pg/mL. Statistical significance was found in comparing liver BDNF sham vs. 1d (p=0.0004).

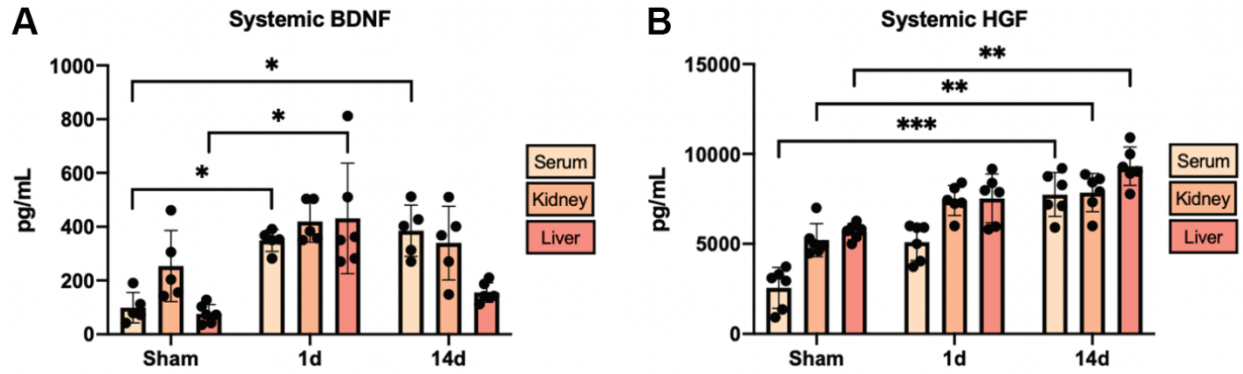


Figure 9: Acute and repeated remote ischemic conditioning confers changes in BDNF and HGF to multiple organ systems. Levels of BDNF (A) and HGF (B) in serum, kidney and liver samples of mice were quantified by ELISA. 18 animals were split into three groups: sham, 1d and 14d animals (n=6 per group). Error bars show SD derived from animals within groups. Kruskal-Wallis test with multiple comparisons was used for statistics. * P-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3.4 Characterizing the interleukin serum profile of animals that underwent single and repeated remote ischemic conditioning

We hypothesized that we would expect to see changes in the serum profile of animals undergoing single or repeated RIC. First, we analyzed the serum for changes in levels of interleukins, which are immune targets that have pleiotropic roles. Serum from three animal groups (sham, 1d and 14d) were analyzed for changes in various interleukin levels. There were eight animals in the sham and 1d groups, and seven animals in the 14d group. Mean value ± SD are described below. The proinflammatory interleukin panel revealed no statistically significant changes across groups. IL-1α levels trended downwards where sham animals averaged 7.28 ± 1.73pg/mL, 1d group averaged 5.70 ± 1.10pg/mL and the 14d group averaged 5.31 ± 1.91pg/mL. Other proinflammatory interleukins tested were: IL-1β (sham 0.11 ± 0.27pg/mL, 1d 0.39 ± 0.81pg/mL, 14d 0.44 ± 0.75pg/mL), IL-6 (sham 2.23 ± 0.35pg/mL, 1d 2.29 ± 1.07pg/mL, 14d 2.27 ± 1.11pg/mL), IL-12p40 (sham 2.34 ± 0.49pg/mL, 1d 2.01 ± 0.33pg/mL, 14d 2.06 ± 0.24pg/mL), IL-12p70 (sham 3.88 ± 2.36pg/mL, 1d 4.51 ± 2.05pg/mL, 14d 3.71 ± 1.02pg/mL), and IL-17 (sham 1.63 ± 0.76pg/mL, 1d 1.39 ± 0.32pg/mL, 14d 1.51 ± 0.37pg/mL). The anti-inflammatory panel revealed no statistically significant changes across groups. IL-10 trended downwards across groups with sham animals averaged at 11.61 ± 1.78pg/mL, 1d animals averaged 8.24 ± 1.45pg/mL and 14d animals averaged at 7.69 ± 2.10pg/mL. Other anti-inflammatory interleukins tested were: IL-4 (sham 0.19 ± 0.03pg/mL, 1d 0.18 ± 0.03pg/mL, 14d 0.19 ± 0.03pg/mL), IL-6 (sham 2.23 ± 0.35pg/mL, 1d 2.29 ± 1.07pg/mL, 14d 2.27 ± 1.11pg/mL), IL-9 (sham 5.99 ± 3.40pg/mL, 1d 7.63 ± 4.33pg/mL, 14d 5.12 ± 2.83pg/mL), and IL-13 (sham 1.17 ± 1.57pg/mL, 1d 0.30 ± 0.34pg/mL, 14d 0.09 ± 0.27pg/mL).

Various other interleukins were also tested with these serum groups. No statistically significant changes were found across groups. IL-16 trended upwards across groups where sham animals averaged 5.95 ± 0.99pg/mL, 1d animals averaged 8.81 ± 0.24pg/mL and 14d animals averaged 9.64 ± 0.55pg/mL. Other interleukins tested were: G-CSF (sham 0.80 ± 0.49pg/mL, 1d 0.65 ± 0.45pg/mL, 14d 0.48 ± 0.32pg/mL), IL-2 (sham 11.18 ± 2.81pg/mL, 1d 9.85 ± 0.95pg/mL, 14d 9.18 ± 1.27pg/mL), IL-3 (sham 0.66 ± 0.24pg/mL, 1d 0.68 ± 0.12pg/mL, 14d 0.57 ± 0.16pg/mL), IL-5 (sham 0.47 ± 0.13pg/mL, 1d 0.45 ± 0.10pg/mL, 14d 0.48 ± 0.15pg/mL), IL-7 (sham 4.58 ± 1.14pg/mL, 1d 4.20 ± 0.88pg/mL, 14d 3.81 ± 0.64pg/mL), IL-11 (sham 2.28 ± 2.16pg/mL, 1d 3.18 ± 6.20pg/mL, 14d 3.21 ± 2.75pg/mL), IL-15 (sham 4.88 ± 1.07pg/mL, 1d 4.71 ± 0.61pg/mL, 14d 4.67 ± 0.37pg/mL), IL-20 (sham 12.64 ± 14.95pg/mL, 1d 10.16 ± 11.78pg/mL, 14d 4.64 ± 8.62pg/mL), and LIF (sham 0.74 ± 0.30pg/mL, 1d 0.76 ± 0.25pg/mL, 14d 0.32 ± 0.14pg/mL).

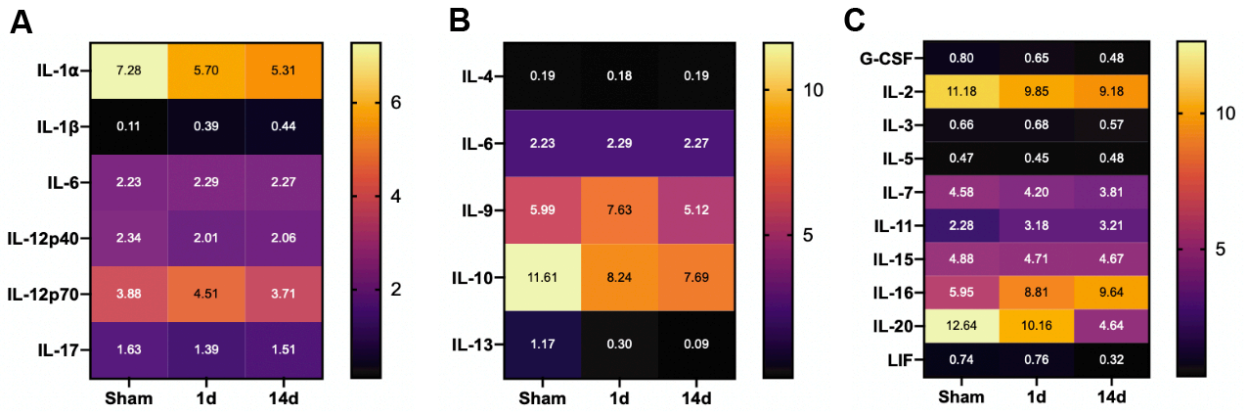


Figure 10: Repeated remote ischemic conditioning induces non-significant changes in levels of serum interleukins. Serum of sham (n=8), 1d (n=8) and 14d (n=7) repeated RIC animals were collected for analysis with proinflammatory IL (A), anti-inflammatory IL (B) and general IL (C) panels. Units for all values are pg/mL.

3.5 Characterizing the CC cytokine and CXC chemokine serum profile of animals that underwent single and repeated remote ischemic conditioning

We also wanted to look for serum changes for CC and CXC chemokines with single and repeated RIC. Serum from three animal groups (sham, 1d and 14d) were analyzed for changes in levels of CC cytokines and CXC chemokines. Data are represented as mean value ± SD. The CC cytokine panel revealed no statistically significant changes across groups. CC cytokines tested were: 6Ckine (sham 3.14 ± 0.92pg/mL, 1d 3.94 ± 0.11pg/mL, 14d 4.45 ± 0.10pg/mL), Eotaxin (sham 6.43 ± 1.21pg/mL, 1d 6.40 ± 1.57pg/mL, 14d 5.80 ± 0.76pg/mL), MCP-1 (sham 15.03 ± 4.35pg/mL, 1d 14.46 ± 3.78pg/mL, 14d 13.37 ± 1.60pg/mL), MCP-5 (sham 36.75 ± 8.35pg/mL, 1d 36.69 ± 6.90pg/mL, 14d 44.59 ± 13.43pg/mL), MDC (sham 1.10 ± 0.29pg/mL, 1d 1.03 ± 0.18pg/mL, 14d 0.89 ± 0.20pg/mL), MIP-1α (sham 17.74 ± 5.67pg/mL, 1d 16.18 ± 3.25pg/mL, 14d 16.88 ± 4.91pg/mL), MIP-1β (sham 17.58 ± 6.42pg/mL, 1d 17.68 ± 2.86pg/mL, 14d 13.43 ± 2.40pg/mL), MIP-3α (sham 1.92 ± 0.46pg/mL, 1d 1.60 ± 0.50pg/mL, 14d 1.53 ± 0.26pg/mL), MIP-3β (sham 9.50 ± 2.48pg/mL, 1d 6.92 ± 2.20pg/mL, 14d 7.90 ± 2.45pg/mL), RANTES (sham 1.69 ± 0.43pg/mL, 1d 1.55 ± 0.16pg/mL, 14d 1.56 ± 0.26pg/mL), and TARC (sham 4.44 ± 1.14pg/mL, 1d 3.48 ± 1.31pg/mL, 14d 4.03 ± 1.12pg/mL).

The CXC chemokine panel revealed no statistically significant changes across groups. CXC chemokines tested were: fractalkine (sham 1.77 ± 0.29pg/mL, 1d 1.68 ± 0.51pg/mL, 14d 1.97 ± 0.51pg/mL), CXCL2 (sham 2.02 ± 1.65pg/mL, 1d 1.47 ± 2.40pg/mL, 14d 1.57 ± 2.34pg/mL), CXCL3 (sham 8.68 ± pg1.94pg/mL, 1d 7.51 ± 2.08pg/mL, 14d 7.86 ± 1.01pg/mL), CXCL9 (sham 0.47 ± 0.21pg/mL, 1d 0.51 ± 0.14pg/mL, 14d 0.68 ± 0.49pg/mL), CXCL10 (sham 8.66 ± 2.31pg/mL, 1d 9.23 ± 1.63pg/mL, 14d 10.84 ± 4.14pg/mL), CXCL12 (sham 1.07 ±

0.16pg/mL, 1d 9.45 ± 0.24pg/mL, 14d 9.37 ± 0.23pg/mL), and MIP-2 (sham 6./mL, 1d 6.98 ± 1.13pg/mL, 14d 5.53 ± 0.97pg/mL).

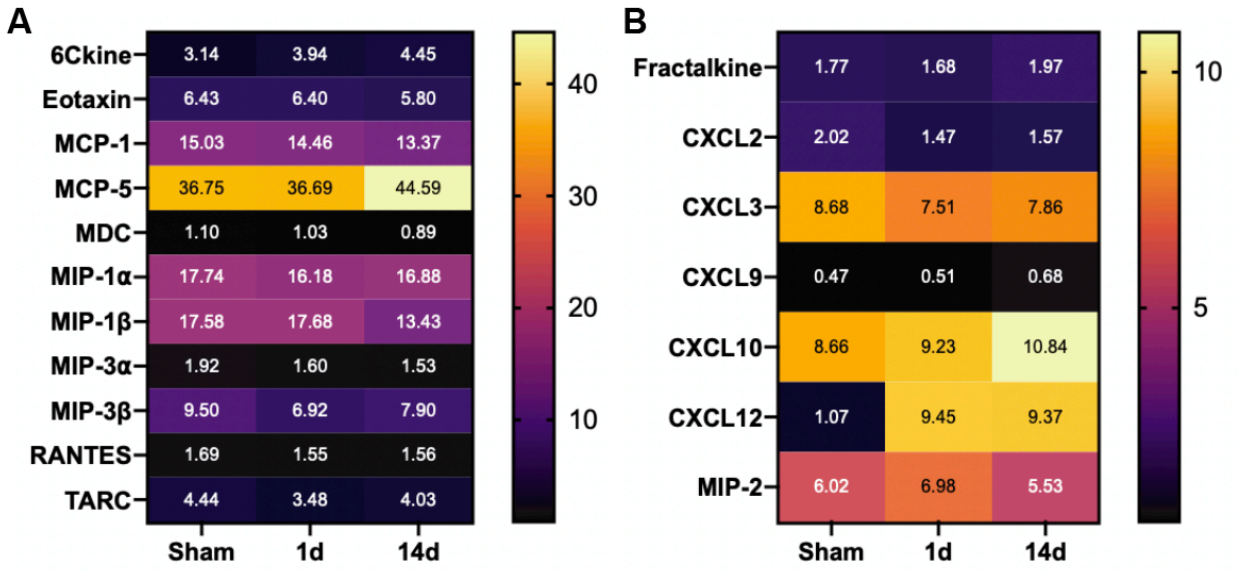


Figure 11: Repeated remote ischemic conditioning induces non-significant changes in levels of CC cytokines and CXC chemokines. Serum of sham (n=8), 1d (n=8) and 14d (n=7) repeated RIC animals were collected for analysis with CC cytokine (A) and CXC chemokine (B) panels. Units for all values are pg/mL.

3.6 Characterizing the growth factor and various target serum profile of animals that underwent single and repeated remote ischemic conditioning

Serum from three animal groups (sham, 1d and 14d) were analyzed for changes in levels of growth factors and select other targets. The growth factor panel revealed no statistically significant changes across groups. Growth factors tested were: amphiregulin (sham 3.65 ± 0.57pg/mL, 1d 2.95 ± 0.61pg/mL, 14d 3.84 ± 1.02pg/mL), angiopoietin-2 (sham 1.53 ± 0.24pg/mL, 1d 1.44 ± 0.18pg/mL, 14d 1.59 ± 0.28pg/mL), EGF (sham 17.11 ± 5.01pg/mL, 1d 9.07 ± 8.76pg/mL, 14d 12.61 ± 7.58pg/mL), FGF-2 (sham 9.98 ± 0.84pg/mL, 1d 8.46 ± 0.64pg/mL, 14d 8.69 ± 1.31pg/mL), HGF (sham 2.69 ± 0.38pg/mL, 1d 2.97 ± 0.80pg/mL, 14d 3.83 ± 0.85pg/mL), TGFβ-1 (sham 6.95 ± 1.38pg/mL, 1d 7.40 ± 1.59pg/mL, 14d 6.51 ± 1.32pg/mL), TGFβ-2 (sham 4.36 ± 1.13pg/mL, 1d 6.67 ± 4.04pg/mL, 14d 4.53 ± 2.59pg/mL),

TGFβ-3 (sham 3.73 ± 0.35pg/mL, 1d 3.52 ± 0.67pg/mL, 14d 3.88 ± 0.93pg/mL), VEGF-A (sham 1.74 ± 0.73pg/mL, 1d 1.70 ± 0.78pg/mL, 14d 1.65 ± 0.97pg/mL), and VEGF-D (sham 2.08 ± 0.40pg/mL, 1d 1.63 ± 0.33pg/mL, 14d 1.83 ± 0.62pg/mL).

Other selected targets were tested with the three serum groups. HSP70 was statistically significant (***) in comparing the sham and 14d group (sham 3.10 ± 1.46pg/mL vs. 14d 8.41 ± 1.55pg/mL, p=0.0006). Within multiple comparisons, the HSP70 1d group (6.98 ± 1.41pg/mL) was not statistically significant compared to either the sham or 14d groups. No other targets were statistically significant across groups. Other targets tested were: endothelin (sham 5.74 ± 0.82pg/mL, 1d 4.72 ± 0.81pg/mL, 14d 4.31 ± 1.07pg/mL), endoglin (sham 3.31 ± 1.46pg/mL, 1d 3.03 ± 1.05pg/mL, 14d 3.12 ± 1.49pg/mL), fas ligand (sham 19.00 ± 5.68pg/mL, 1d 11.98 ± 4.71pg/mL, 14d 14.05 ± 11.01pg/mL), follistatin (sham 2.95 ± 0.42pg/mL, 1d 2.43 ± 0.48pg/mL, 14d 2.70 ± 0.68pg/mL), IFNβ-1 (sham 4.84 ± 0.83pg/mL, 1d 3.19 ± 0.10pg/mL, 14d 4.09 ± 0.96pg/mL), IFNγ (sham 5.30 ± 1.61pg/mL, 1d 4.66 ± 0.86pg/mL, 14d 4.89 ± 1.25pg/mL), leptin (sham 14.82 ± 2.05pg/mL, 1d 12.34 ± 1.52pg/mL, 14d 14.29 ± 3.78pg/mL), PGF-2 (sham 18.10 ± 4.36pg/mL, 1d 15.38 ± 3.76pg/mL, 14d 17.31 ± 4.37pg/mL), prolactin (sham 1.46 ± 0.71pg/mL, 1d 2.11 ± 0.21pg/mL, 14d 3.02 ± 0.31pg/mL), sALK-1 (sham 5.46 ± 1.22pg/mL, 1d 3.91 ± 1.34pg/mL, 14d 4.53 ± 1.19pg/mL), TIMP-1 (sham 5.38 ± 0.69pg/mL, 1d 5.52 ± 1.06pg/mL, 14d 4.95 ± 0.78pg/mL), and TNFα (sham 0.80 ± 0.20pg/mL, 1d 0.85 ± 0.14pg/mL, 14d 0.73 ± 0.12pg/mL).

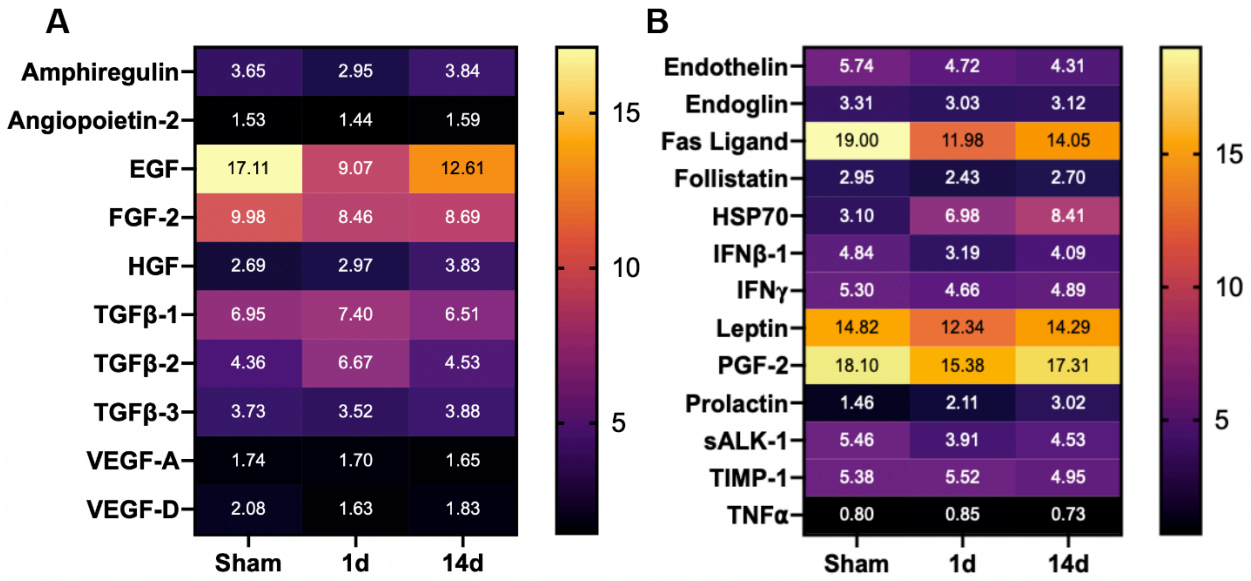


Figure 12: Repeated remote ischemic conditioning induces changes in levels of growth factors and other proteins. Serum of sham (n=8), 1d (n=8) and 14d (n=7) repeated RIC animals were collected for analysis with a growth factor (A) and a varied target (B) panel. Units for all values are pg/mL.

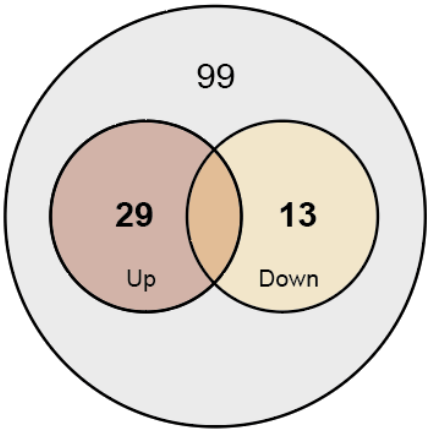
3.7 Differential expression of RNA in the spinal cord transcriptome with single remote ischemic conditioning

Transcriptomic changes in the spinal cord of animals that received one (1d) or fourteen days (14d) of RIC were evaluated using bulk RNA sequencing. Spinal cords of animals that received 1d RIC (n=8) were compared to animals that received one day of the sham intervention (n=8). The results are summarized in Figure 13. The expression of 99 genes were altered based on statistical significance in comparing the 1d RIC and 1d sham spinal cords. Further bioinformatic analysis incorporating the inclusion and exclusion criteria (Methods 2.9) revealed that there were 29 upregulated genes and 13 downregulated genes. The top ten genes that were most significantly upregulated based on ln fold change were: ANO10 (4.9051), DNAH7 (4.9229), KCNMA1 (4.9904), KMT2C (5.1345), Foxn3 (5.1369), ASPH (5.4486), NF2 (5.6985), Cyp2d22 (5.7963), EPB41L3 (5.9080) and ATRNL1 (5.9336). The top ten genes were most significantly downregulated based on ln fold change were: FBXO9 (-6.8027), APLP2 (-6.6666),

LONRF2 (-6.3880), KATNAL1 (-5.5647), SNAP23 (-4.7054), NCOA2 (-4.2530), NRXN1 (-1.1633), ADAP1 (-1.0633), ZMYM4 (-0.7350) and SLC1A2 (-0.7004).

The genes that were differentially expressed after the inclusion and exclusion criteria were submitted to Metascape (https://metascape.org/gp/index.html#/main/step1) for further analysis. The GO pathways associated with the upregulated dataset were "MAPK family signaling cascades" (R-MMU-5683057) and "neuron projection morphogenesis" (GO:0048812). Three pathways were associated with the downregulated data set: "signaling by Rho GTPases" (R-MMU-194315), "head development" (GO:0060322) and "neurotransmitter transport" (GO:0006836). Further pathway analysis elucidated specific targets that are associated with select pathways. "Neuron projection morphogenesis" comprises the upregulation of *USP31*, *Dst*, *CTNND2*, and *EPB41L3*. "MAPK signaling" comprises the upregulation of *RANBP9*, *SEPTIN7*, *SPRED1*, and *Foxn3*. "Neurotransmitter transport" comprises the downregulation of *SYT2*, *SLC1A2*, *NRXN1*, and *SNAP23*.

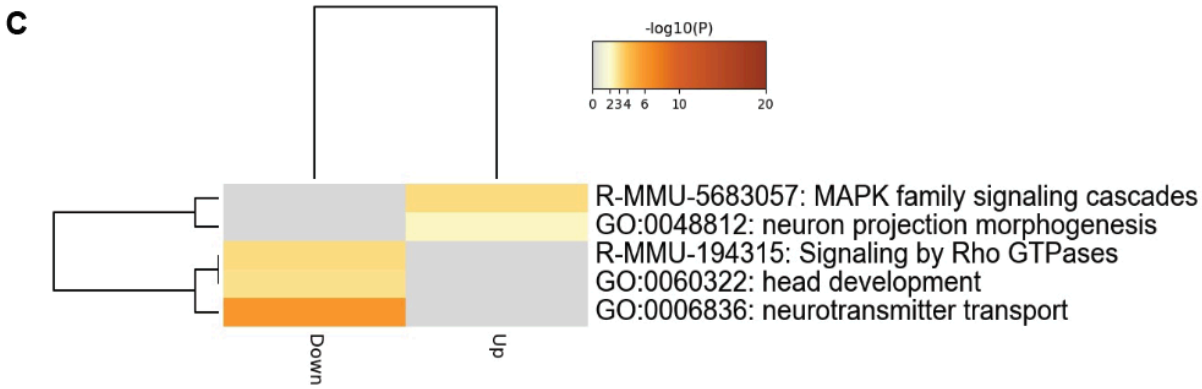
A



B

Upregulated		Downregulated	
Gene	ln Fold Change	Gene	ln Fold Change
ANO10	4.9051	FBXO9	-6.8027
DNAH7	4.9229	APLP2	-6.6666
KCNMA1	4.9902	LONRF2	-6.3880
KMT2C	5.1345	KATNAL1	-5.5647
Foxn3	5.1369	SNAP23	-4.7054
ASPH	5.4486	NCOA2	-4.2530
NF2	5.6985	NRXN1	-1.1633
Cyp2d22	5.7963	ADAP1	-1.0633
EPB41L3	5.9080	ZMYM4	-0.7350
ATRNL1	5.9336	SLC1A2	-0.7004

C



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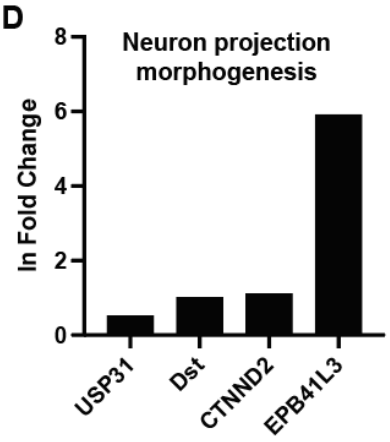

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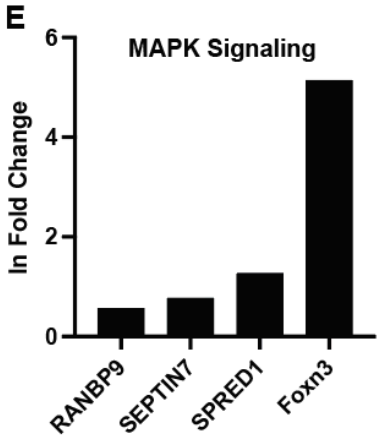

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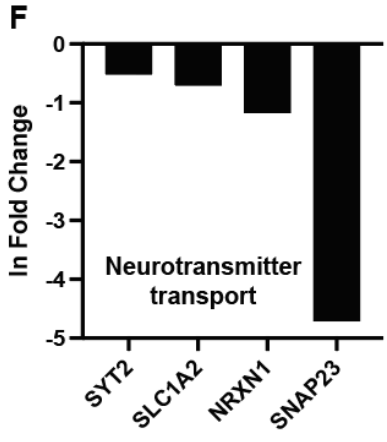


Figure 13: Bulk RNA sequencing of mouse spinal cord that underwent 1 day of remote ischemic conditioning. 1d (n=8) of RIC compared to sham controls (n=8). (A) 99 genes were altered in the dataset. After inclusion and exclusion criteria, 29 of those genes were significantly upregulated and 13 were significantly downregulated. (B) A list of the ten highest upregulated and downregulated genes from the dataset. (C) Gene ontology pathway analysis of the significantly altered genes. (D) Pathways of interest that include the associated genes.

3.8 Differential expression of RNA in the spinal cord transcriptome with repeated remote ischemic conditioning

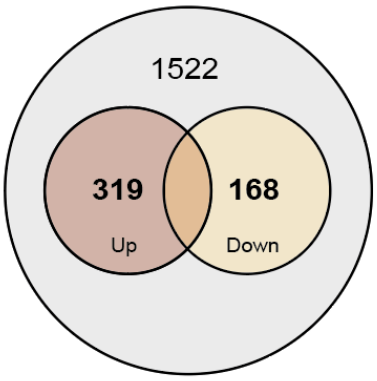
Spinal cords of animals that received 14d RIC (n=8) were compared to animals that received fourteen days of the sham intervention (n=8). The results are summarized in Figure 14. The expression of 1522 genes were altered based on statistical significance in comparing the 14d RIC and 14d sham spinal cords. Further bioinformatic analysis incorporating the inclusion and exclusion criteria (Methods 2.9) revealed that there were 319 upregulated genes and 168 downregulated genes. The top ten genes that were most significantly upregulated based on ln fold change were: ABCA2 (7.0588), KMT2D (6.0582), KCNMA1 (6.0452), EPB41L3 (5.9133), UBR5 (5.8843), ATRNL1 (5.8428), SYT1 (5.6711), GRIN1 (5.6348), KMT2C (5.4866) and PLEC (5.4490). The top ten genes were most significantly downregulated based on ln fold change were: RBFOX1 (-7.3262), GSE1 (-6.7033), APLP2 (-6.5958), KATNAL1 (-6.4245), LONRF2 (-6.2077), TACR1 (-5.8185), CACNA1E (-5.6381), NAP1L1 (-5.5259), DAB2IP (-5.4579) and ATP13A3 (-5.3331).

The genes that were differentially expressed after the inclusion and exclusion criteria were submitted to Metascape (https://metascape.org/gp/index.html#/main/step1) for further analysis. The GO pathways associated only with the upregulated data set were: “endocytosis” (hsa04144), “protein-protein interactions at synapses” (R-HSA-6794362), “negative regulation of cellular component organization” (GO:0051129), and “myelination” (GO:0042552). The GO pathways associated only with the downregulated data set were: “retrograde endocannabinoid signaling” (hsa04144), “protein localization to the plasma membrane” (GO:0072659), “regulation of cell morphogenesis” (GO:0022604), and “regulation of plasma membrane bounded cell projection organization” (GO:0120035). Certain GO pathways were variably

associated with both the upregulated and downregulated datasets. Of these pathways, the ones that were more differentially expressed in the upregulated compared to downregulated datasets were: “response to alcohol” (GO:0097305), “regulation of cellular localization” (GO:0060341), “regulation of circadian rhythm” (GO:0042752), “regulation of supramolecular fiber organization” (GO:1902903), and “cell junction organization” (GO:0034330). The pathways that were more differentially expressed in the downregulated compared to the upregulated datasets were: “positive regulation of catabolic processes” (GO:0009896), “regulation of ion transport” (GO:0043269), “protein catabolic processes” (GO:0030163), “vesicle-mediated transport” (R-HSA-5653656), “modulation of chemical synaptic transmission” (GO:0050804), “synaptic signaling” (GO:0099536), and “neuronal system” (R-HSA-112316).

Further pathway analysis elucidated specific targets that are associated with select pathways. “Myelination” comprises the upregulation of *SCN8A*, *CNTNAP1*, *ARHGEF10*, *MOBP*, *MBP*, *NTRK2*, *KLHL8*, *PLEC*, and *EPB41L3*. “Protein-protein interaction at synapses” comprises the upregulation of *DLG2*, *PPFIA4*, *DLGAP1*, *PTPRS*, *SHANK1*, *EPB41*, *EPB41L1*, *GRIN1*, and *SYT1*. “Cell morphogenesis” comprises the downregulation of *EPS8*, *LPAR1*, *CAPZB*, *SYT2*, *CDC42SE1*, *ARPC2*, *ZMYM4*, *MAP3K13*, *CUX1*, *PALM*, and *AKAP8*. Analysis of transcriptional regulatory networks associated with upregulated genes revealed upregulation of TP53 and SIRT1.

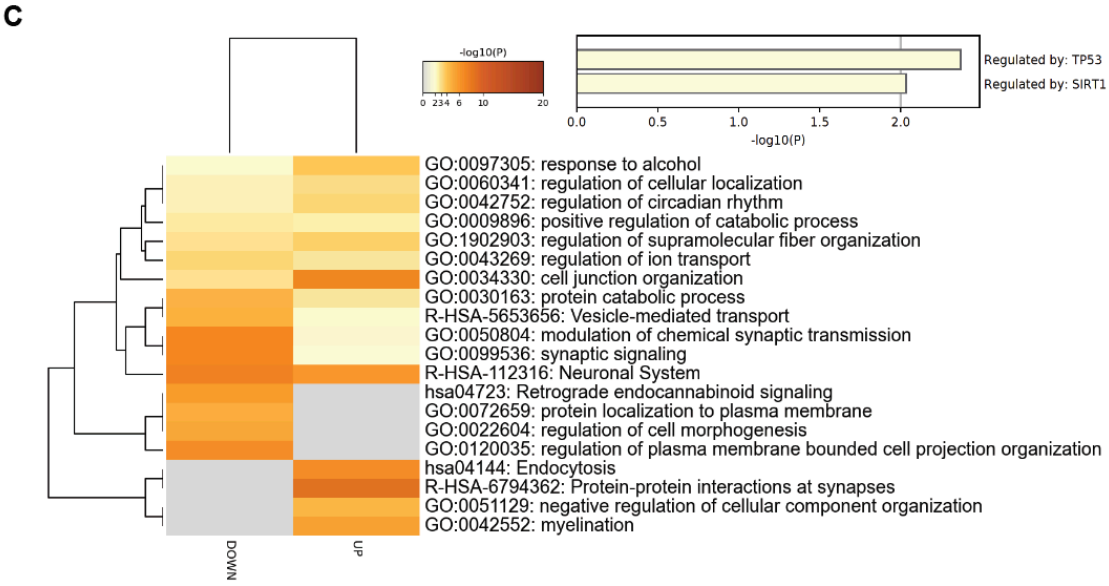
A



B

Upregulated		Downregulated	
Gene	ln Fold Change	Gene	ln Fold Change
ABCA2	7.0588	RBFOX1	-7.3262
KMT2D	6.0582	GSE1	-6.7033
KCNMA1	6.0452	APLP2	-6.5958
EPB41L3	5.9133	KATNAL1	-6.4245
UBR5	5.8843	LONRF2	-6.2077
ATRNL1	5.8428	TACR1	-5.8185
SYT1	5.6711	CACNA1E	-5.6381
GRIN1	5.6348	NAP1L1	-5.5259
KMT2C	5.4866	DAB2IP	-5.4579
PLEC	5.4490	ATP13A3	-5.3331

C



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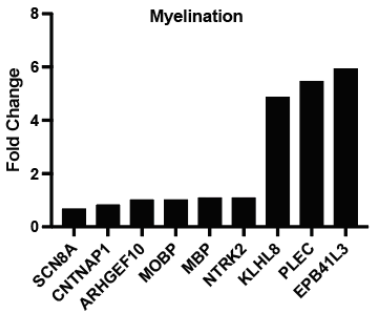

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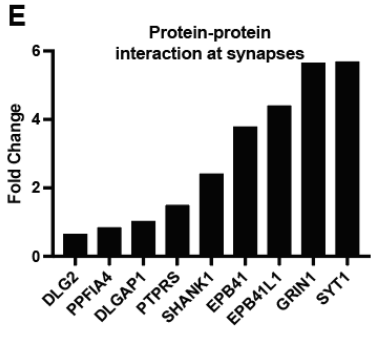

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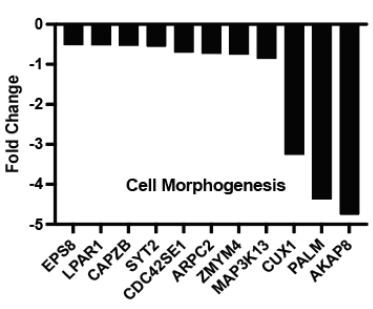


Figure 14: Bulk RNA sequencing of mouse spinal cord that underwent 14 consecutive days of repeated remote ischemic conditioning. 14d (n=8) of repeated RIC compared to sham controls (n=8). (A) 1522 genes were altered in the dataset. After inclusion and exclusion criteria, 319 of those genes were significantly upregulated and 168 were significantly downregulated. (B) A list of the ten highest upregulated and downregulated genes from the dataset. (C) Gene ontology pathway analysis of the significantly altered genes. (D) Pathways of interest that include the associated genes.

3.9 Differential expression of proteins in the spinal cord proteome with repeated remote ischemic conditioning

Spinal cords of animals that received 14d RIC were compared to animals that received fourteen days of the sham intervention. Five animals were used in each group. The proteomic results are summarized in Figure 15. The expression of 1862 proteins were altered based on statistical significance in comparing the 14d RIC and 14d sham spinal cords. Further bioinformatic analysis incorporating the inclusion and exclusion criteria (Methods 2.9) revealed that there were 223 upregulated proteins and 79 downregulated proteins. The top ten proteins that were most significantly upregulated based on H/L ratio were: G6pdx (142.33), Rps12 (75.595), Atp1a3 (35.779), Baz1a (21.646), Yars (21.271), Hsp90aa1 (20.586), Rtn4 (19.417), Arhgdia (19.151), Cntrl (18.731), and Rps24 (14.535). The top ten proteins that were most significantly downregulated based on H/L ratio were: Ccdc166 (0.018361), Cluh (0.022611), Tceal1 (0.023874), Tchp (0.030324), Col1a2 (0.058229), Abat (0.074121), Acly (0.078197), Kat8 (0.081406), Atp6v1a (0.086983), and Col1a1 (0.089466).

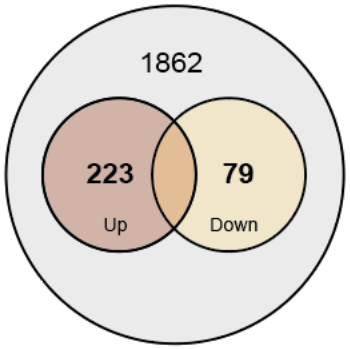
The proteins that were differentially expressed after the inclusion and exclusion criteria were submitted to Metascape (https://metascape.org/gp/index.html#/main/step1) for further analysis. The GO pathways associated only with the upregulated data set were: “metabolism of amino acids and derivatives” (R-HSA-71291) and “cell part morphogenesis” (GO:0032990). Other GO pathways were associated with both the upregulated and downregulated datasets. The pathways that were more differentially expressed in the upregulated compared to downregulated datasets were: “cellular responses to stress” (R-HSA-2262752), “nervous system development” (R-HSA-9675108), “carbon metabolism” (hsa01200), “pathways of neurodegeneration – multiple diseases” (hsa05022), “signaling by Rho GTPases” (R-HSA-194315), “neutrophil

degranulation" (R-HSA-6798695), "parkin-ubiquitin proteasomal system pathway" (WP2359), "negative regulation of cellular component organization" (GO:0051129), "regulation of cell projection organization" (GO:0031344), "HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand" (R-HSA-3371497), "membrane organization" (GO:0061024), "response to inorganic substance" (GO:0010035), "organelle localization" (GO:0051640), "head development" (GO:0060322), "regulation of cellular localization" (GO:0060341), "ATP metabolic process" (GO:0046034), and "generation of precursor metabolites and energy" (GO:0006091). The pathway that was more differentially expressed in the downregulated compared to upregulated datasets was "ribose phosphate metabolic process" (GO:0019693). Further pathway analysis elucidated specific targets that are associated with the "cell part morphogenesis" pathway. This pathway is comprised of the upregulation of *Actbl2*, *Ptpn11*, *Cdk5*, *Gap43*, *Map1b*, *Crmp1*, *Cnp*, *S100b*, *Cyfip1*, *Nefl*, *Nfasc*, *Cntnap1*, *Lama2*, *Ncam1*, *Tnr*, *Cntn1*, *Stxbp1*, and *Hsp90aa1*. Analysis of transcriptional regulatory networks associated with upregulated genes were Pparg. Transcriptional regulatory networks associated with the downregulated genes were Sp1, Runx2, and Ep300.

We validated targets NCAM1, Nfasc and HSP90 from the repeated proteomics data set with ELISA using the same animal groups (n=6 per group). NCAM1 measured sham 9217.17 ± 4356.58pg/mL, 1d 12889 ± 2729.13pg/mL, and 14d 16252.20 ± 3405.17pg/mL. Statistical significance was revealed in comparing sham vs. 14d ($p=0.0175$). Nfasc measured sham 2.50 ± 0.99ng/mL, 1d 3.98 ± 1.39ng/mL, and 14d 5.67 ± 2.21ng/mL. Statistical significance was revealed in comparing sham vs. 14d ($p=0.0171$). HSP90 measured sham 3.52 ± 0.95ng/mL, 1d 6.12 ± 0.82ng/mL, and 14d 8.57 ± 1.28ng/mL. Statistical significance was revealed in comparing

sham vs. 14d ($p=0.0016$). All three validating markers matched the trends seen in the proteomics dataset.

A



B

Upregulated		Downregulated	
Protein	H/L Ratio	Protein	H/L Ratio
G6pdx	142.33	Ccdc166	0.018361
Rps12	75.595	Cluh	0.022611
Atp1a3	35.779	Tceal1	0.023874
Baz1a	21.646	Tchp	0.030324
Yars	21.271	Col1a2	0.058229
Hsp90aa1	20.586	Abat	0.074121
Rtn4	19.417	Acly	0.078197
Arhgdia	19.151	Kat8	0.081406
Cntrl	18.731	Atp6v1a	0.086983
Rps24	14.535	Col1a1	0.089466

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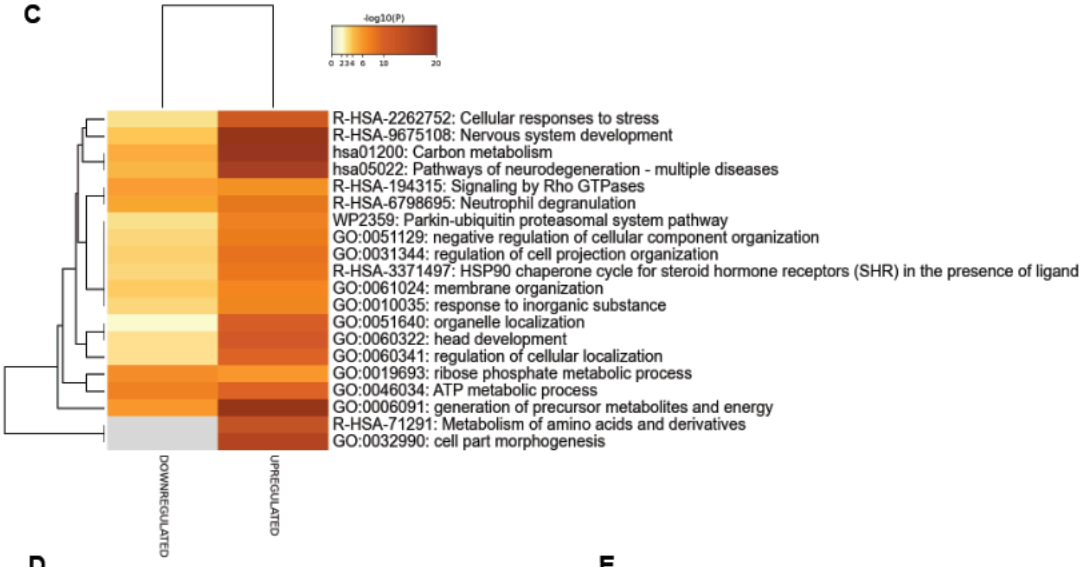

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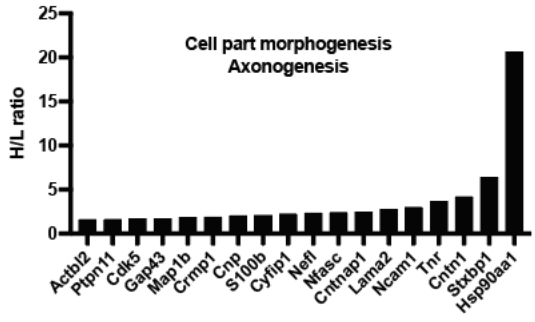

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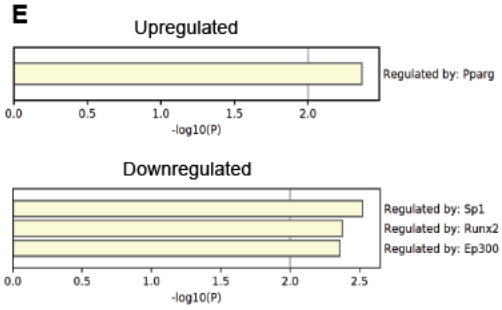

Figure 15: Shotgun proteomics of mouse spinal cord that underwent 14 consecutive days of repeated remote ischemic conditioning. Proteomic analysis of sham animals (n=5) compared to 14d of repeated RIC (n=5). (A) 1862 proteins were altered in the dataset. After inclusion and exclusion criteria, 223 of those proteins were significantly upregulated and 79 were significantly downregulated. (B) A list of the ten highest upregulated and downregulated proteins from the dataset. (C) Gene ontology pathway analysis of the significantly altered proteins. (D) Pathways of interest that include the associated proteins. (E) Upstream regulation of the upregulated and downregulated proteins.

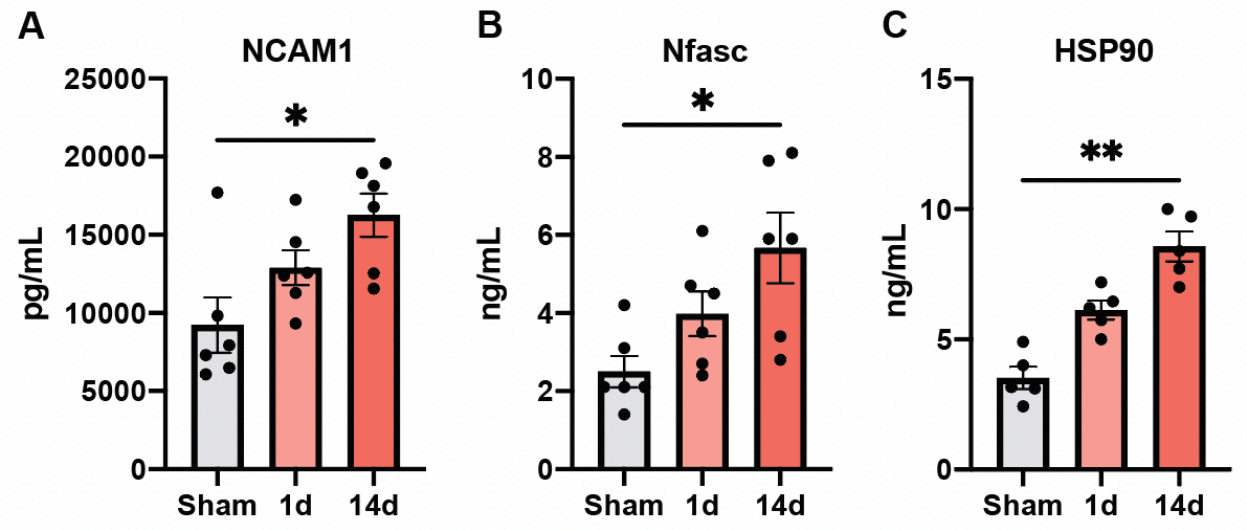

Figure 16: ELISA validation of proteomics data with selected targets NCAM1, neurofascin, and heat shock protein 90. Comparison of sham (n=6), 1d RIC (n=6), and 14d RIC (n=6) to validate the proteomic data. The selected validation targets using ELISA: (A) neural cell adhesion molecule 1 (NCAM1), (B) neurofascin (Nfasc), and (C) heat shock protein 90 (HSP90). Error bars show SD derived from samples within groups. Statistical analysis using a Kruskal-Wallis test with multiple comparisons. (*) P-value < 0.05, (**) P-value < 0.01.

3.10 Upregulation of gene ontology pathway myelination is associated with the repeated remote ischemic conditioning proteomic and transcriptomic data sets

Comparison of the 14d proteomic and 14d RNA sequencing datasets reveals eight matched targets (<2%). The six targets that were upregulated across both datasets were: plectin (PLEC, 5.4490 fold change and 1.777 H/L ratio), dynamin 1 (DNM1, 1.5114 ln fold change and 3.2084 H/L ratio), myelin basic protein (MBP, 1.0753 ln fold change and 3.2037 H/L ratio), contactin associated protein 1 (CNTNAP1, 0.8184 ln fold change and 2.42 H/L ratio), contactin-binding protein 2 (CTTNBP2, 0.5405 ln fold change and 2.8297 H/L ratio), and brevican (BCAN, 0.6475 ln fold change and 1.8392 H/L ratio). The two targets that were downregulated across both datasets were: nucleosome assembly protein 1 like 1 (NAP1L1, -5.5259 ln fold change and 0.28731 H/L ratio) and semaphorin 3A (SEMA3A, -0.5755 ln fold change and 0.45507).

These eight targets were submitted to Metascape (https://metascape.org/gp/index.html#/main/step1) for further analysis. The GO pathways associated with these targets were: "myelination" (GO:0042552), "nervous system development" (R-HSA-9675108), and "brain development" (GO:0007420). The "myelination" pathway comprises the upregulation of CNTNAP1, MBP and PLEC.

A

	Target	ln Fold Change	H/L Ratio
Up	PLEC	5.4490	1.777
	DNM1	1.5114	3.2084
	MBP	1.0753	3.2037
	CNTNAP1	0.8184	2.42
	CTTNBP2	0.5405	2.8297
	BCAN	0.6475	1.8392
Down	NAP1L1	-5.5259	0.28731
	SEMA3A	-0.5755	0.45507

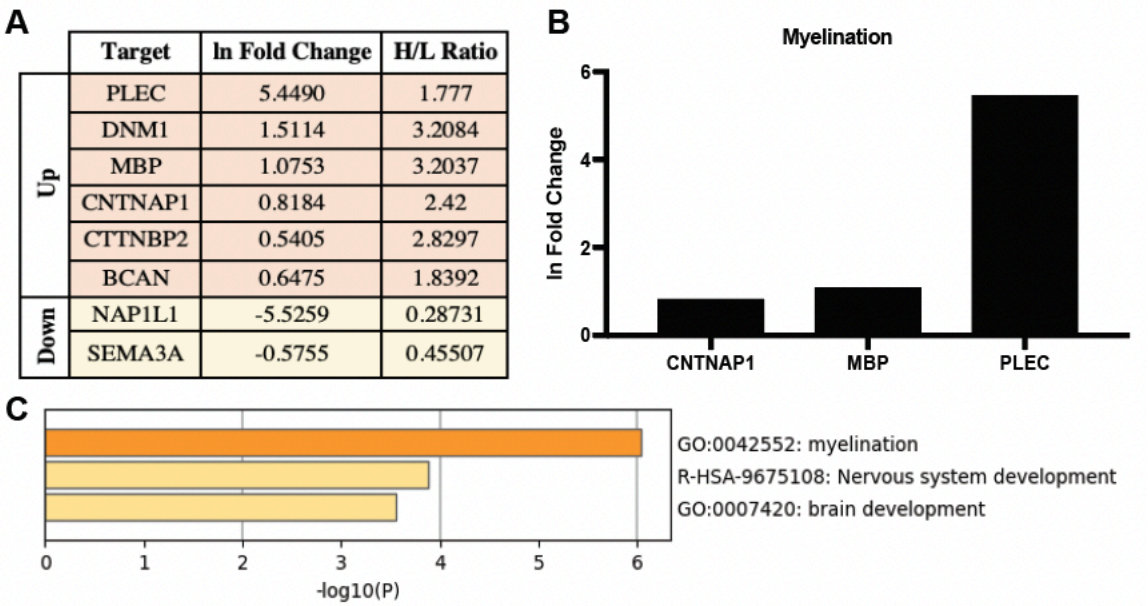

Figure 17: Comparison of 14-day repeated remote ischemic conditioning proteomics and bulk RNA sequencing data. (A) Targets that overlapped between the 14 day and 14-day proteomic datasets. (B) The gene ontology "myelination" pathway was associated with both the 14-day RNA and proteomic datasets. (C) Other gene ontology pathways that were associated with both the 14-day RNA and proteomic datasets.

3.11 Repeated remote ischemic conditioning does not change lesion size in animal model of multiple sclerosis

The experimental design of lysolecithin-induced demyelination is shown in Figure 18. In comparing sham (n=5) and 14-day RIC (n=5) groups, lesion appearance with eriochrome cyanine staining did not change. The sham animal group's lesion area averaged 76173 ± 2915μm^2 and the 14-day group's lesion area was an average of 77304 ± 3947μm^2. The

difference between group means was 1131 ± 2194. Using an unpaired t test, no significant difference between the group's means was determined (p value = 0.6201).

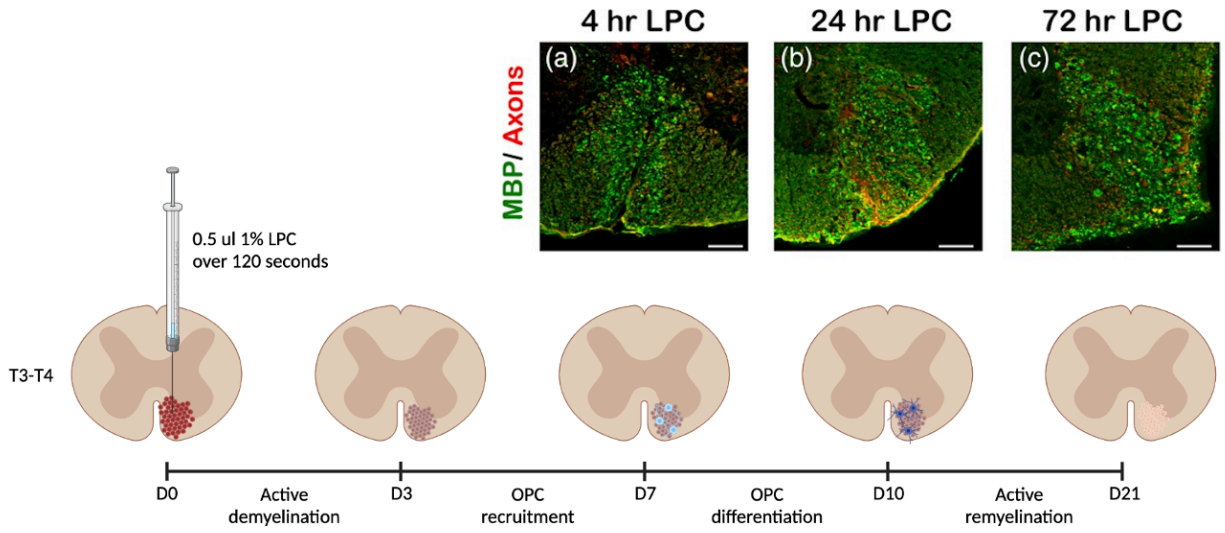

Figure 18: Overview of LPC-induced demyelination model. Bottom schematic was created by author using BioRender (https://biorender.com/). Staining of MBP (green) and axons (red) change rapidly over time following LPC injury (modified from Plemel et al., 2018).

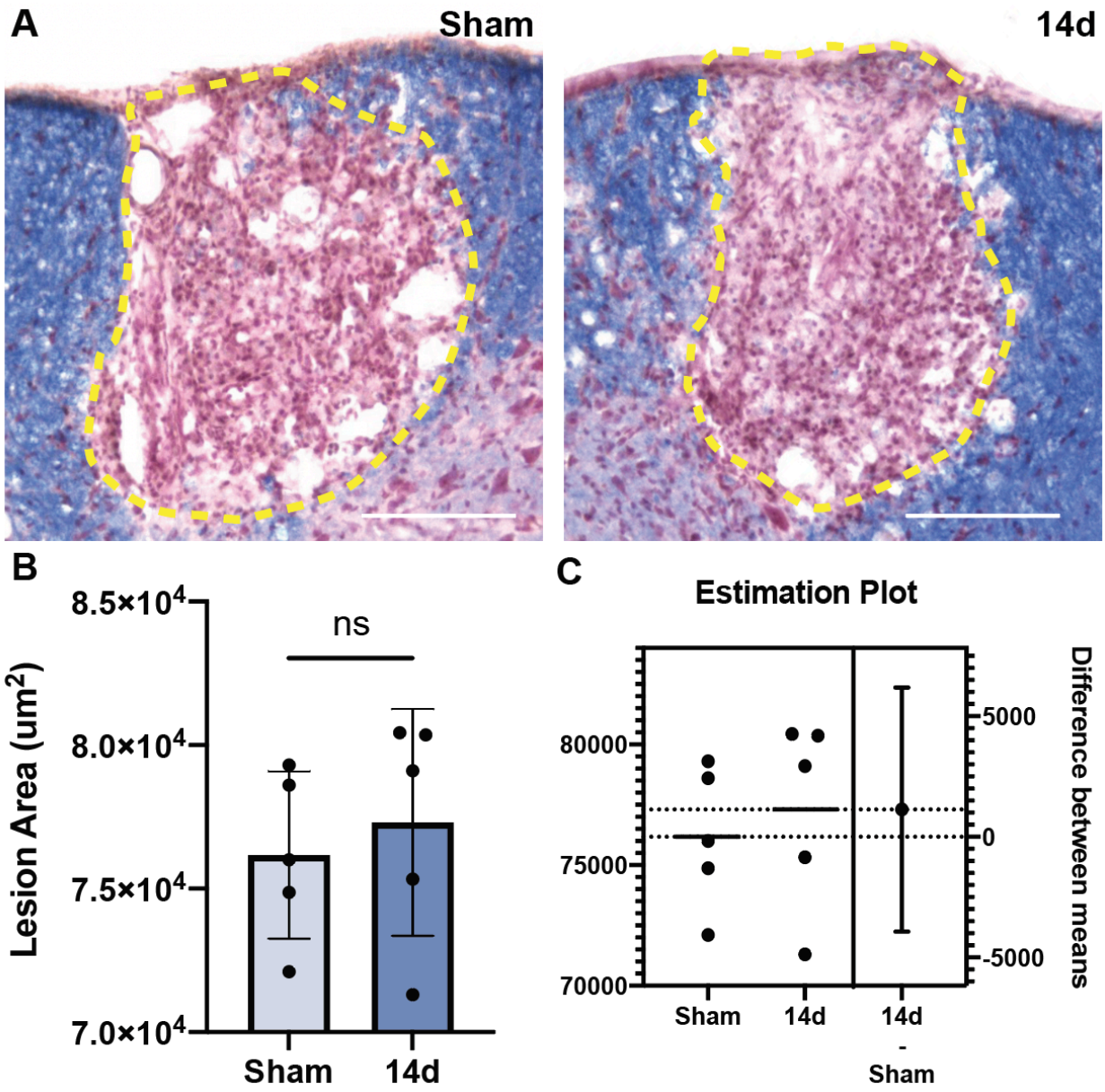

Figure 19: Lesion area quantification of animals that underwent sham or 14 days of repeated remote ischemic postconditioning. (A) Eriochrome cyanine staining used to identify demyelinated lesion (pink). Yellow dashed line represents the lesion region of interest. (B) Quantification of lesion area comparing sham (n=5) and 14-day repeated RIC groups (n=5). (C) Estimation plot of the difference between means of panel B. Error bars show SD derived from animals within groups. Unpaired t test was used for statistics.

3.12 Repeated remote ischemic conditioning alters the lesion microenvironment in an animal model of multiple sclerosis

Figure 20 quantifies positive oligodendrocyte lineage marker (Olig2), proliferation marker (ki67), and nuclear (DAPI) staining in the LPC-induced lesion at day 14 post-injury. In the sham group (n=6), 12.33 ± 2.81% of Olig2+ cells were also proliferating (ki67+). In the 14-day group (n=6), 10.83 ± 2.32% of Olig2+ cells were also ki67+. An unpaired t test determined that there was no significant difference between these means. However, quantification of proliferating cells (DAPI+/ki67+) revealed a 1.42-fold change increase in the repeated RIC group compared to sham.

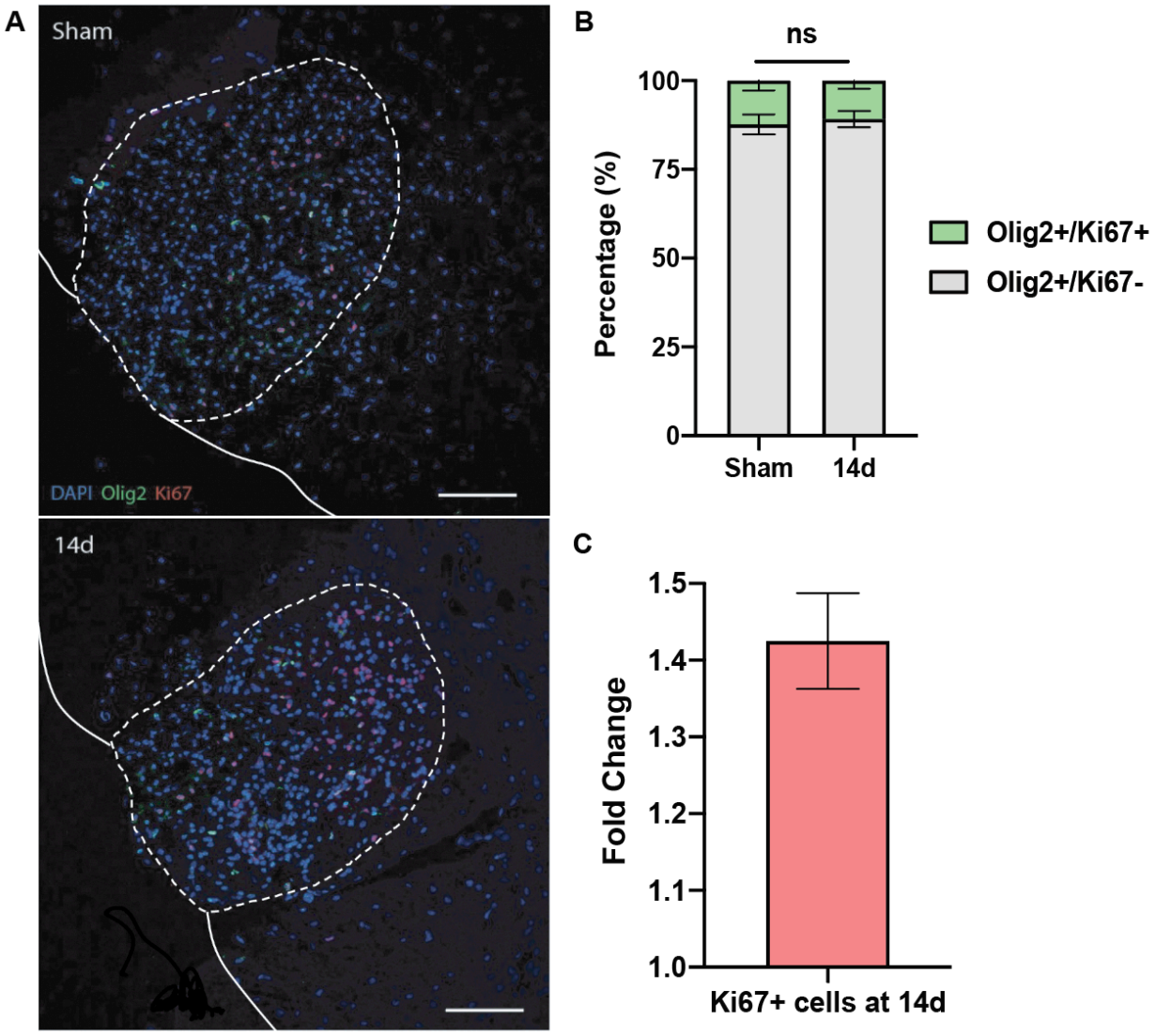

Figure 20: Evaluating oligodendrocyte proliferation in sham and 14-day postconditioned lesions. (A) T3-T4 spinal cords stained with nuclear DAPI (blue), oligodendrocyte lineage (Olig2), and proliferation marker (ki67) stains. Images were taken with a 25x lens. (B) Quantification of double positive Olig2+ and ki67+ cells in lesion region of interest. Cells that were Olig2+ and ki67- were also quantified and overall represented as a percentage. (C) Quantification of ki67+ cells in the lesion region of interest as a fold change compared to sham. Scale bars at 25x represent 100µm. Error bars show SD derived from animals within groups. Unpaired t test was used for statistics.

Based on our findings in the RNA sequencing and proteomics experiments, we selected targets to evaluate in the lesion with repeated remote ischemic conditioning. Figure 21 quantifies positive neurofascin (Nfasc) staining in the LPC-induced lesion at day 14 post-injury. In the sham group (n=8), Nfasc staining covered 6.01 ± 1.33% of the ROI. The 14d group (n=8)

showed 9.43 ± 1.39% positive Nfasc staining in the ROI. Nfasc staining was confined to the core of the lesions, with little to no positive signal in the peripheral area. The difference between means of the sham group compared to the 14d group is 3.43 ± 0.68.

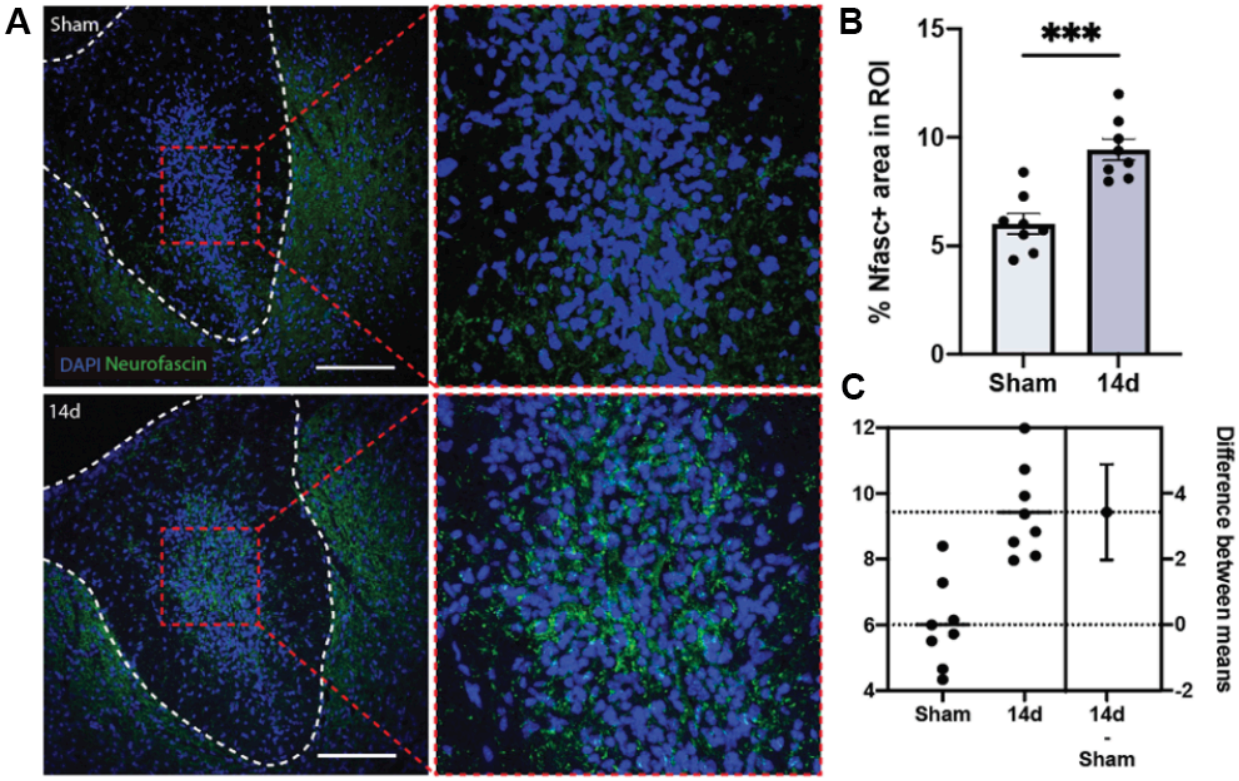

Figure 21: Neurofascin staining in the lesion core of animals that underwent sham or 14 days of repeated remote ischemic postconditioning. (A) T3-T4 spinal cords stained with nuclear DAPI (blue) and neurofascin (green). Images were taken with 25x and 40x lens. (B) Quantification and (C) difference in means of positive stained area within the lesion. Scale bars at 25x represent 100µm. Each group contained eight animals. Error bars show SEM derived from animals within groups. Unpaired t test was used for statistics. *** P-value < 0.001.

Figure 22 quantifies positive plectin (Plec) staining in the LPC-induced lesion at day 14 post-injury. Plec+ staining was spread throughout the entire lesion ROI. In the sham group (n=6), Plec+ staining averaged 0.75 ± 0.17cells/µm^2. The 14d group (n=6) showed 1.01 ± 0.14cells/µm^2 that were Plec+. The difference between means of the sham group compared to the

14d group is 0.26 ± 0.09. An unpaired t test comparing the groups revealed a significant difference (p value = 0.0156).

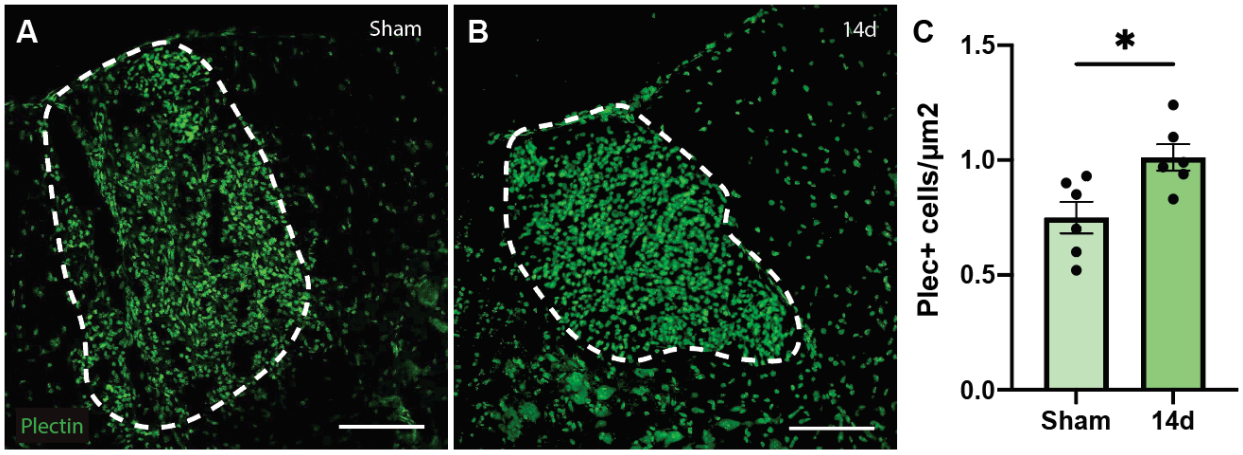

Figure 22: Plectin staining in sham and 14-day postconditioned lesions. (A) T3-T4 spinal cords stained with plectin (Plec) (green). Images were taken with 25x (B) Quantification of Plec+ staining by counting positively stained cells per area. Each group contained six animals. Error bars show SD derived from animals within groups. Unpaired t test was used for statistics. * P-value < 0.05.

Figure 23 quantifies positive myelin basic protein (MBP) and neurofilament heavy chain (NFH) staining in the LPC-induced lesion at day 14 post-injury. In the sham group (n=8), MBP staining covered 12.95 ± 2.95% of the ROI. The 14d group (n=8) showed 16.85 ± 4.62% positive MBP staining in the ROI. Mann-Whitney statistical testing revealed a significant difference between these groups (p value = 0.0463). In the sham group, NFH staining covered an average of 47.75 ± 6.43% of the ROI and the 14-day group averaged 49.10 ± 11.32%. Using the same statistical test, no significant difference between these means was found.

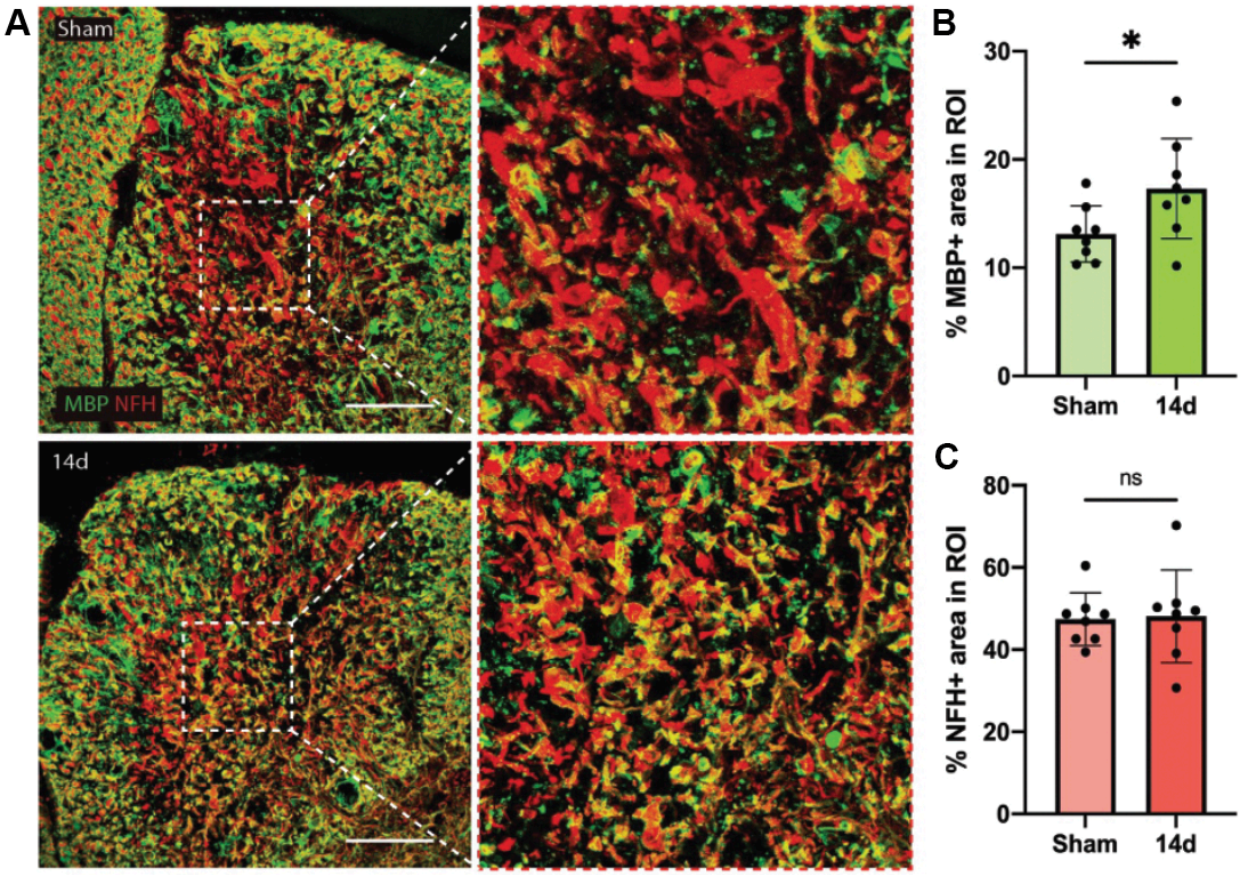

Figure 23: Myelin basic protein and neurofilament heavy-chain staining in sham and 14-day postconditioned lesions. (A) T3-T4 spinal cords stained with myelin basic protein (MBP) (green) and neurofilament heavy chain (NFH) (red). Images were taken with 25x and 40x lens. (B) Quantification of MBP+ staining in the lesion region of interest. (C) Quantification of NFH+ staining in the lesion region of interest. Each group contained eight animals. Scale bars at 25x represent 100µm. Error bars show SD derived from animals within groups. A Mann-Whitney test was used for statistics on both data sets. * P-value < 0.05.

Chapter 4: Discussion

Research regarding the use of RIC has been developed in various basic science and clinical research fields. Repurposing the intervention for use in other neurodegenerative diseases may provide an accessible adjunct treatment for patients. Most treatment options for pwMS focus on targeting the inflammatory aspects of the disease pathophysiology. Research on RIC has been largely focused on the cardiovascular effects of the intervention. Literature has not previously described the effects of RIC in the CNS. There are supported hypotheses as to how RIC may be able to aid white matter repair and protection in a diseased context. Further research to guide treatment options for repair of white matter damage is needed to enhance patient treatment. This thesis intended to investigate the potential of RIC in an animal model of MS. Specifically, the purpose of this thesis was to develop a safe and feasible model of repeated RIC in an animal model of MS and to explore changes due to repeated RIC in white matter lesions in this model.

Repeated RIC is tolerable intervention that has shown positive outcomes in clinical trials across multiple fields (Aimo et al., 2015; Landman et al., 2019). RIC upregulates molecules involved in angiogenesis, hypoxia and antioxidant pathways (Da Silva Costa et al., 2016; Ren et al., 2019; Yu et al., 2022). A major shortcoming in this field of research is a consensus on the optimal temporal variant for the intervention. This shortcoming is likely also linked to the mystery behind the mechanisms that underlie RIC. Using the EF5 assay we tested the safety of repeated remote ischemic conditioning in the mouse model. In this assay, EF5 is a cyclic nitroimidazole that selectively binds to hypoxic cells to form adducts (Koch & Evans, 2015). Hypoxic conditions cause an increase in nitroreductase activity on the EF5 marker, which causes the formation of reactive cellular nucleophiles. The activated EF5 nucleophile then selectively

binds to the hypoxic cell adducts (Bergeron et al., 1999). A second Alexa Fluor 488 fluorescent-conjugated ELK3-51 antibody binds to the EF5+ cells to qualitatively assess hypoxia in tissues. We first wanted to answer whether the conditioning paradigm we selected was causing any hypoxic damage to the leg tissue of the mouse. We compared two conditioning paradigms: animals who received four cycles of five minutes of ischemia followed by five minutes of reperfusion (5i/5r x4) vs. animals who received 40 minutes of uninterrupted ischemia. 40 minutes of uninterrupted ischemia produced a positive EF5 signal in the leg tissue, indicating detectable tissue hypoxia. The muscle of animals that received our conditioning paradigm did not demonstrate any positive hypoxic signature. A drawback with the EF5 assay is the inability to quantify the signal, which was previously indicated. However, this experiment was designed as a proof-of-concept that did not require quantification. The experiment results demonstrated that the five minutes of ischemia followed by reperfusion was not causing significant damage to the tissue.

To further validate this novel intervention, we tracked physiological changes of mice undergoing RIC. At rest, temperature and visual observation of the hind paw was noted. Every five minutes during the inflation/deflation of the cuff, temperature and visual observations were also noted. The hind paw temperature prior to the intervention was typically around 25°C. When the sphygmomanometer was inflated to maximum pressure, the blood flow was occluded to the limb. The temperature of the cuffed hind paw dropped in temperature and transitioned from a pink to pale gray color. As expected, when the cuff was deflated after five minutes, the blood flow to the paw was restored which reversed the color change. Upon reperfusion the temperature of the hind paw also restored to the baseline temperature. These observable changes indicate significant change in physiology due to RIC.

Previous research involving RIC has proposed many mechanisms and molecules involved in the intervention signaling. RIC is also an intervention that is relatively broad and can confer protective effects over multiple different organ systems (Brevoord et al., 2012; Hu et al., 2016; Yasin et al., 2014). We wanted test this by evaluating changes in levels of target molecules in different systems that may be induced with acute and repeated RIC. BDNF is a trophic factor involved in neuroplasticity, learning and memory (Miranda et al., 2019). Increased BDNF has been demonstrated in animal models of RIC (Ramagiri & Taliyan, 2017) and ischemic preconditioning (Neumann et al., 2015). We tested levels of BDNF in serum, kidney and liver of animals that received the sham intervention, one application of RIC and repeated RIC for 14 days. Serum level of BDNF was significantly upregulated in both the single and repeated RIC groups when compared to sham animals. Liver level of BDNF was significantly upregulated in the acute RIC group. After fourteen days of repeated RIC, BDNF level in the liver returned to a level comparable to the sham group. Kidney level of BDNF was not statistically significant across any groups. However, there was an upward trend in the single and repeated groups when compared to the sham group. Research has indicated that RIC induces both an early and late phase of protection, which may play a role in these findings (Loukogeorgakis et al., 2005).

HGF is a well-characterized trophic factor with a variety of functions. Signaling through the HGF receptor MET has been implicated in mediating neuroinflammation and angiogenesis in neurodegenerative disease (Desole et al., 2021). HGF has also been implicated in signaling recovery from ischemia-reperfusion injury (Liu et al., 2014). This molecule is also a cardioprotective factor that induces cardiomyocyte proliferation and differentiation (Gallo et al., 2014). We tested levels of HGF in serum, kidney and liver of animals that received the sham intervention, one application of RIC and repeated RIC for 14 days. HGF was significantly

upregulated in comparing sham and repeated conditioning across serum, kidney and liver groups. No significance was found in any group when comparing sham and single conditioning groups.

Characterizing global changes to the serum profile due to chronic RIC has not been previously described. Understanding the changes due to RIC may further elucidate potential molecular signaling targets and provide a better understanding for future therapeutic uses. We tested serum of animals grouped into sham, single conditioning and 14 days of repeated conditioning groups. We tested the serum with interleukin, chemokine, cytokine, growth factor, and various other target panels. This is important because changes in cytokine levels due to RIC could have implications in the mechanisms, safety, and potential benefit of RIC in inflammatory conditions such as MS.

Interleukins are expressed by leukocytes and play a large, broad role in immune cell differentiation, cell proliferation, maturation, and migration (Cuneo & Autieri, 2012). Most interleukins can be separated into anti-inflammatory or proinflammatory groups based on their response to different stimuli. Many proinflammatory interleukins, such as IL-1β, are known to be systemically elevated throughout the pathogenesis of MS (Palle et al., 2017). Thus, targeting interleukin levels in MS has been an attractive strategy for therapeutic research. We sought out testing levels of common pro-inflammatory interleukins in the serum of animals that received a single or repeated RIC intervention. Compared to sham animals, IL-1α trended downwards with single and repeated RIC. However, these values were not statistically significant. IL-1β did not demonstrate any trending difference between groups. Signaling of the IL-1 family has been heavily implicated in induction and pathophysiology of MS. IL-1 signaling drives induction and neuroinflammation in EAE (Musella et al., 2020). We also tested the serum for two dimers of IL-12: IL-12p40 and IL-12p70. IL-12 is a heterodimeric cytokine that play a part in driving Th1

cells contribution to the pathogenesis of MS (Yang et al., 2011). We did not find any statistical significance or trends when comparing the levels of these dimers across all groups. IL-17 was another target evaluated based on its implications in the pathogenesis of MS. CD8+ T cells found in active MS lesions produce IL-17 and contribute to the white matter degeneration (Lückel et al., 2019). Il-17 levels in serum were comparable across all groups. The last "pro-inflammatory" cytokine tested in the serum was IL-6. IL-6 can demonstrate both pro- and anti-inflammatory characteristics depending on the environment and signaling. In the context of MS, IL-6 signaling supports resistance to T cell regulation and may worsen the disease (Schneider et al., 2013). Levels of IL-6 in the serum of all groups was comparable, with no trending or significant differences.

In conjunction with proinflammatory interleukins, their anti-inflammatory counterparts are also important in MS. We wanted to test whether single or repeated RIC could contribute to a change in levels of various anti-inflammatory interleukins. IL-4 administration into the cerebrospinal fluid (CSF) of EAE animals shows improved neurophysiological and clinical recovery (Butti et al., 2008). However, in our study IL-4 levels did not change with single or repeated RIC. IL-9 decreases immune activation and promotes anti-inflammatory signaling in MS (Donninelli et al., 2020). IL-9 serum level increased after one cycle of RIC but did not maintain elevated levels within the repeated group. The trend upwards after one intervention was not statistically significant. In MS, low IL-10 levels have been associated with higher clinical disability and MRI lesion load (Petereit et al., 2003). There is a downwards trend of serum levels of IL-10 with single and repeated RIC. However, this finding was not statistically significant. The last anti-inflammatory cytokine tested was IL-13. IL-13 is theorized to play a neuroprotective role in the CNS and in the context of MS (Rossi et al., 2011). Levels of serum

IL-13 decreased with single and repeated RIC when compared to sham animals. This decrease was not statistically significant.

We also tested the serum for interleukins that have functions beyond mediating inflammation but may be implicated in neuroprotection and neurodegenerative diseases. Single and repeated RIC does not change the levels of circulating β common chain cytokines: G-CSF, IL-3, or IL-5. High level of pleiotropic cytokine G-CSF is associated with severe exacerbation of MS (Openshaw et al., 2000). G-CSF has also been implicated in the resolution of inflammatory episodes. IL-3 and IL-5 share common structural and functional properties with G-CSF (Dougan et al., 2019). IL-2 signaling has a paradoxical function in MS, implicated in differential induction of autoimmune responses and tolerance (Peerlings et al., 2021). Compared to sham serum, single and repeated RIC had a downward trend of IL-2 levels. This was not statistically significant. IL-7 levels have been implicated in MS treatment prognosis. Low levels of serum in pwMS may indicate a better response to interferon therapy (Lee et al., 2011). In our experiment, IL-7 did not change with single or repeated conditioning when compared to sham. IL-11 has been implicated in the exacerbation of disease in animal models of MS (Zhang et al., 2019). IL-15 has also been implicated in proinflammatory processes of CNS and peripheral immune cells (Laurent et al., 2021). RIC did not alter serum levels of IL-11 or IL-15. IL-16 serves an immunomodulatory effect in the context of MS through its CD4 receptor (Skundric et al., 2015). IL-16 trended upwards in the single RIC group and even moreso in the repeated group. IL-20 deficient mice show improved EAE scoring and increased angiogenesis-related activity through VEGF (Dayton et al., 2021). IL-20 showed a slight decrease in serum levels after a single application of RIC. This trend downwards was amplified in the repeated RIC group. Both trends in IL-16 and IL-20

were not statistically significant. LIF modulates the immune response in MS by inhibiting Th17 cell differentiation (Vela et al., 2016). Single and repeated RIC did not alter levels of serum LIF.

To further investigate serum profile changes, we tested the same serum groups with various chemokine and cytokine panels. CC chemokines are secreted proteins that act as chemotactics for monocytes and lymphocytes through signaling with their CC receptor (Hughes & Nibbs, 2018). CC chemokines and cytokines have been heavily implicated in the pathogenesis and treatments in MS (Cui et al., 2020). 6Ckine holds angiostatic properties and acts as a chemoattractant with T cells (Soto et al., 1998). Single and repeated RIC did not induce any change in levels of serum 6CKine compared to sham animals. Eotaxin and RANTES have displayed neuroprotection in animal models of neurodegenerative disease (Chandra et al., 2016; Huber et al., 2018). We did not see any significant trends of change with RIC regarding the serum levels of eotaxin or RANTES. MCPs are chemotactic proteins that hold a neuroprotective role in the CNS and peripheral immune system. MCP-1 may be implicated in neuroprotection against ischemia-reperfusion injury in animal models and in vitro (Jin et al., 2015). MCP-1 and MCP-5 are also target genes for HIF-1α during the hypoxia response (Mojsilovic-Petrovic et al., 2007). We found that levels of MCP-1 were not changed across groups. However, levels of serum MCP-5 trended upwards with single and repeated RIC. Different MIP subtypes play a role in neuroinflammation in injury, and serve as potential therapeutic targets (Ciechanowska et al., 2020; DiSabato et al., 2016). We found that serum levels of MIP-1α, MIP-1β, MIP-3α, and MIP-3β did not change across single or repeated RIC groups when compared to sham animals. MDC is a proinflammatory chemokine that is necessary for the inflammatory response through its receptor chemokine receptor type 4 (Richter et al., 2014). Levels of serum MDC did not change across the groups tested. The last CC chemokine that we tested was TARC. TARC is

known to play a role in the inflammatory response in an allergic reaction. Specifically, TARC has been implicated in driving the Th2-driven inflammatory reaction (Vestergaard et al., 2004). We did not find any changes in levels of TARC in serum due to single or repeated RIC.

We also tested the serum groups with a panel of CXC chemokines. In general, CXC chemokines are chemotactic for neutrophils and interact with CXC receptor subtypes (Graves & Jiang, 1995). Fractalkine (CX3CL1) has been shown to increase reparative capabilities during demyelination and regulate CD4+ cell migration in MS (Blauth et al., 2015). CXCL2 and CXCL3 are similar in that they are both proinflammatory and promote recruitment and adhesion of neutrophils at sites of injury. Across all serum groups, fractalkine, CXCL2, and CXCL3 did not differ in values. CXCL9 and CXCL10 both interact with CXCR3 to produce signaling. CXCL9 is a chemoattractant for T cells, while CXCL10 is a regulator of leukocyte trafficking. Levels of CXCL9 were unchanged across all serum groups. CXCL12 is involved in homeostatic processes like embryogenesis (Dhaiban et al., 2020). CXCL10 and CXCL12 trended upwards with single and repeated conditioning when compared to sham. However, these findings were not statistically significant. MIP-2 has been implicated in the mediation of inflammation, but not within the context of MS (Qin et al., 2017). However, levels of MIP-2 in serum were unaltered.

Broad changes in levels of pleiotropic molecules have been reported with RIC (Zhou et al., 2018). We sought out to assess whether we could observe changes in serum levels of various growth factors with single or repeated RIC. We selected two panels of growth factors and other proteins for testing that have pleiotropic effects in angiogenesis, neuroprotection or inflammatory pathways. Amphiregulin is part of the epidermal growth factor (EGF) family. EGF and EGF family members have demonstrated neuroprotective effects among many functions such as cell proliferation, differentiation and growth (Zhan et al., 2015). EGF in serum was non-significantly

downregulated in single and repeated RIC groups compared to sham. Amphiregulin did not show any altered levels in serum. Fibroblast growth factor-2 (FGF-2) is a mitogenic factor that has also demonstrated neuroprotection in EAE (Rottlaender et al., 2011). FGF-2 levels in all serum groups were unchanged. Angiopoietin-2 is a neurotrophic factor that can inhibit apoptosis, reduced inflammation and promote angiogenesis (Yin et al., 2019). VEGF-A and VEGF-D are also significant mediators of angiogenesis. RIC has been shown ability to induce angiogenesis in-vitro and in-vivo, further inducing protection of multiple organ systems (Hummitzsch et al., 2021; Ma et al., 2013). No angiogenesis-related molecule (angiopoietin, VEGF-A, VEGF-D) was altered in the serum of all groups. TGFβ cytokines are multifunctional and play roles in neuroprotection via anti-inflammatory, anti-apoptotic and angiogenesis signaling (Dobolyi et al., 2012). We did not see any significant changes in serum levels of TGFβ-1, TGFβ-2, or TGFβ-3.

Several other notable target molecules were of interest in exploring the effects of single and repeated RIC. Endothelin and endoglin are two peptides involved in angiogenesis and neuroprotection. Endothelin plays a role in protection of retinal ganglion cells and increased expression of this peptide has also been demonstrated as a biomarker of poor recovery in optic neuritis in pwMS (Castellazzi et al., 2019; Kusaka et al., 2022). Endoglin may promote angiogenesis through activation of the vascular endothelial cells and induce protective pathways in hypoxic cells (Kopczyńska & Makarewicz, 2012). However, endoglin and endothelin were not altered with single or repeated RIC. Fas ligand is a member of the TNF family that may play a role in regulation of nerve repair (Li et al., 2018). Single and repeated RIC groups showed a downwards trend when compared to control, but this was not a statistically significant finding. Low levels of immune cell follistatin in pwMS has been proposed as a potential contributor to remyelination failure (Urshansky et al., 2011). Conversely, high levels of prolactin promote

myelin repair (Zhornitsky et al., 2012). However, both serum levels of prolactin and follistatin were unaltered with single or repeated RIC. Certain interferons, such as IFNγ and IFNβ-1, have been shown to inhibit remyelination in the CNS (Lin et al., 2006; Trebst et al., 2007). Serum levels of IFNγ and IFNβ-1 were unaltered with single and repeated RIC when compared to controls. Leptin is a secreted hormone that can promote the proliferation of OPCs and aid in remyelination in animal models of demyelination (Matoba et al., 2017). Tissue inhibitor of metalloproteinases-1 (TIMP-1) plays a similar role in driving OPC differentiation. Conversely, prostaglandin F-2 (PGF-2) interaction with receptor PGF-2α may be a mediator in OPC death (Takahashi et al., 2013). Nevertheless, leptin, TIMP-1 and PGF-2 did not show any significant changes between control, single RIC and repeated RIC groups. TNFα is a major cytokine that plays a significant role in a multitude of different biological processes. In the context of MS, TNFα can regulate immune dysregulation, demyelination, and neuroinflammation, among many other roles (Fresegna et al., 2020). TNFα serum levels were not significantly altered with the application of single or repeated RIC. The final target we analyzed was heat shock protein 70 (HSP70), which has immunoregulatory characteristics in MS (Mansilla et al., 2012). RIC has been previously shown to increase HSP70 in motor neurons and be neuroprotective in spinal cord ischemia (Danková et al., 2021). In MS, HSP70 has been proposed to play immunomodulatory and cytoprotectant roles (Mansilla et al., 2012). HSP70 was significantly upregulated with repeated conditioning compared to the control animal group. Single RIC trended upwards compared to sham but was not statistically significant.

Upon demonstrating minor changes in the serum profile of animals treated with single and repeated RIC which were not very impressive, we wanted to use an unbiased and comprehensive method to evaluate changes to the mouse spinal cord on a transcriptomic level.

We evaluated two timepoints in line with our previous data: single (1 day) and repeated (14 days) RIC. Significantly upregulated and downregulated genes with a single day of RIC were also used in pathway analysis. The neuron projection morphogenesis pathway, consisting of *USP31*, *Dst*, *CTNND2*, and *EPB41L3*, was significantly upregulated with single RIC. These genes play a role in neuron morphogenesis but also may serve other neuroprotective functions as well. The Catenin Delta 2 (CTNND2) gene can play various roles in facilitating oligodendrocyte lineage differentiation (Pooyan et al., 2022). Dysregulated *CTNND2* was also shown to play a role in neurodevelopmental disease progression (Maussion et al., 2015). Erythrocyte Membrane Protein Band 4.1 Like 3 (EPB41L3) has been demonstrated as a tumor suppressor gene in subtypes of cancer (Zeng et al., 2018). Of interest, *EPB41L3* has also been shown to contribute to the maintenance of myelin by stabilizing membrane proteins at paranodal junctions (Cifuentes-Diaz et al., 2011). MAPK signalling-associated genes were also significantly upregulated after a single cycle of RIC. The Forkhead Box N3 (FOXN3) gene was the fifth highest upregulated gene in the single RIC dataset and most highly upregulated gene associated with MAPK signaling. Significant downregulation of *FoxN3* is associated with tumor progression in fifteen different types of cancer (Kong et al., 2019). While cancer pathophysiology is quite unique, there are systems involved that overlap with white matter neurodegenerative diseases, such as immunology and inflammatory pathways. Upregulation of genes such as *FoxN3*, *CTNND2*, and *EPB41L3* after single RIC may be serving as a "priming" effect against the potential for future damage. Anoctamin 10 (ANO10) is the most significantly upregulated gene in the single RIC data set. *ANO10* mutations are linked to being the primary cause for autosomal recessive cerebellar ataxia, a neurodegenerative disorder that inhibits coordination and muscle movement (Renaud et al., 2014). To our knowledge, there is no

currently published literature that links ANO10 to remyelination or MS. Dynein Axonemal Heavy Chain 7 (DNAH7) was the second most upregulated gene in the data set. Mutations in *DNAH7* are implicated in testicular and colorectal cancers, but no association with white matter protection or neurodegenerative disease (Gao et al., 2022; Yang et al., 2022). Synaptosome associated protein 23 (SNAP23) was the fifth highest downregulated gene in the single RIC dataset. It was also the highest downregulated gene in the neurotransmitter transport-associated pathway. In peripheral nerve regeneration models, knocking out *SNAP23* inhibits neurite outgrowth after injury (Lin et al., 2017). However, *SNAP23* also plays roles in apoptosis, autophagy, and myogenesis (Feng et al., 2018; Gentile et al., 2022). It is unclear what the downregulation of *SNAP23* due to RIC means in the context of white matter damage but may be associated with one of the gene's other functions. Amyloid precursor-like protein 2 (APLP2) was the second highest downregulated gene in the dataset. *APLP2* has been shown to modulate myelin regeneration after injury in animal models (Truong et al., 2019). However, the role of downregulated *APLP2* due to RIC is not well understood. Taken together, a single application of RIC induces changes to the mouse spinal cord transcriptome. There are some indications that certain signaling pathways are being upregulated in anticipation of an injury, but these genes require further research in order to thoroughly understand these results.

We also wanted to examine and compare the transcriptomic changes in the mouse spinal cord with repeated RIC. After 14 consecutive days of daily RIC, mouse spinal cords underwent the same evaluation for transcriptomic changes from the previously described experiment. The tissue produced a more robust set of significantly upregulated and downregulated genes. After clustering analysis, the altered datasets produced signaling pathways that contained a cluster of associated genes. In the context of MS and neuroprotection, we highlighted three pathways that

were of interest to us: myelination, protein-protein interaction at the synapse, and cell morphogenesis. The latter pathway contained significantly downregulated genes while the other two were upregulated. Two genes in the myelination pathway were highly upregulated and within the top 10 highest upregulated genes evaluated in the transcriptomic library: *PLEC* and *EPB41L3*. Plectin (PLEC) is a structural protein that has pleiotropic functions involving many different cell types. In the context of MS, *PLEC* is highly expressed in Schwann cells and therefore may play a role in white matter repair or production (Fuchs et al., 2009). Plectin's interaction with dystrophin and dystroglycan may also play a role in OPC differentiation into myelinating OLs in the CNS (Potokar & Jorgačevski, 2021). Plectin knockout mice have shown myelin sheath deformations and loss of intermediate cytoskeleton structure (Fuchs et al., 2009). *EPB41L3* was highly expressed in both the single and repeated RIC experiments. The elevation of *EPB41L3* with repeated conditioning clustered with other myelination-associated upregulated genes. As aforementioned, *EPB41L3* carries immunosuppressant and myelin maintenance properties. The Neurotrophic Receptor Tyrosine Kinase 2 (NTRK2) gene was also significantly upregulated and associated with the myelination pathway. Activation of this receptor promotes myelin repair in the CNS in animal models (Fletcher et al., 2018). Contactin-associated protein 1 (CNTNAP1) gene was also a myelination-associated gene that was upregulated with repeated RIC. *CNTNAP1* codes for a cell adhesion molecule that has cytoskeletal and white matter functions. Mutations in *CNTNAP1* can cause hypomyelination neuropathies (Mehta et al., 2017). Taken together, the upregulation of specific myelination-associated genes with repeated RIC may be able to prime the nervous system for future demyelination. Another pathway of interest that contained many upregulated genes was the protein-protein interaction at the synapse cluster. The upregulated genes in this pathway are involved in the maintenance of the synapse to ensure

that neuronal signaling is efficient. A gene from that pathway, Erythrocyte Membrane Protein Band 4.1 (EPB41), has been identified as a hub protein for neurodegenerative diseases (Li et al., 2014). Protein Tyrosine Phosphatase Receptor Type S (PTPRS) is another protein-protein synapse interaction-associated gene that has other functions, such as axonogenesis and axon targeting in development (Uetani et al., 2006). Many genes associated with cell morphogenesis were found to be downregulated in the data set. Cell morphogenesis is the process of cell development in which there is growth and differentiation to form tissues (Gilbert, 2000). Cell morphogenesis and myelination are linked in the sense that OPCs must undergo morphogenetic changes to allow for myelination during development and remyelination after injury. Bone morphogenetic proteins also play a role in inhibiting remyelination in MS (Eixarch et al., 2017). One of the downregulated genes from this pathway, Mitogen-Activated Protein 3-Kinase 13 (MAP3K13), may also play a role in myelination. Elevated *MAP3K13* demonstrated a reduction in myelination after traumatic brain injury in an animal model (Welsbie et al., 2019). Taken together, repeated RIC induces both upregulation and downregulation of various biological processes. Many of these processes that are altered are directly or indirectly linked to myelination and myelin maintenance.

Many upregulated genes in the repeated RIC dataset are also linked through regulation by *SIRT1* and *TP53*. Sirtuin 1 (SIRT1) has been described as a key epigenetic regulator of metabolism (Elibol & Kilic, 2018). Induction of autoimmune demyelination in animal models upregulates *SIRT1* expression in OPCs (Prozorovski et al., 2019). It has also been shown that *TP53* encodes protein p53 that maintains myelin integrity (Molina et al., 2011). However, this experiment was explored in the context of white matter volume in patients with schizophrenia and has not yet been described in traditionally demyelinating disease. The most upregulated gene

among the entire dataset was ATP-Binding Cassette Subfamily A Member 2 (ABCA2). The encoded protein is mainly localized to OLs and are hypothesized to play a role in lipid functioning. In an *ABCA2* knockout model, myelin composition was altered when compared to wild-type mice. However, there were no abnormalities with OL differentiation and myelin function (Sakai et al., 2007). Altogether, repeated RIC induces transcriptomic changes to the CNS that can affect many signaling pathways.

From the transcriptomic data, we see significant changes to multiple pathways which supports the idea that RIC is a broadly functioning intervention. We also wanted to assess whether these changes were detectable at the proteomic level. In order to see the most robust changes, we selected the repeated RIC paradigm. As we expected, many pathway-clustered proteins from the tested library were significantly downregulated and upregulated with repeated RIC. Many upregulated proteins were associated with the axonogenesis pathway. Interestingly, Heat Shock Protein 90kDa-α (Hsp90aa1), was the highest upregulated protein from the axonogenesis pathway and the sixth highest protein overall. During our examination of serum markers with repeated RIC, HSP70 was one of the only significantly upregulated targets. HSPs are conserved chaperone proteins that can play significant roles during stress or injury (Turturici et al., 2014). Induction of HSPs is seen in demyelinated lesions in white matter, hypothesized to play a protective role (Peferoen et al., 2015). Contactin 1 (Cntn1) is a cell adhesion protein that is also part of the axonogenesis-associated upregulated proteins. Cntn1 also facilitates organization of myelinated axons during remyelination after injury in MS (Kalafatakis et al., 2021). Neural Cell Adhesion Molecule 1 (Ncam1), also found upregulated in the dataset, has been explored as a potential biomarker in CSF of pwMS (Ziliotto et al., 2019). It's progressive increase in tissues of pwMS have led researchers to also hypothesize that Ncam1 may play a role in regulation of

remyelination after injury (Massaro, 2002). CNTNAP1 was found to be upregulated in both the proteomic and transcriptomic datasets. As aforementioned, CNTNAP1 dysfunction is commonly associated with hypomyelinating neuropathies. Neurofascin (Nfasc) is a pleiotropic protein that plays a role in nervous system development. Nfasc also interacts with CNTN1 in an adhesion complex that maintains paranodal myelin-axon junctions (Chataigner et al., 2022). Independently, OL-derived Nfasc also regulates myelin production in the CNS (Klingseisen et al., 2019). Based on the literature, we chose three targets to validate these proteomic findings: Ncam1, Nfasc, and HSP90. Altogether, many of the upregulated axonogenesis-related proteins have concurrent functions in remyelination, myelin maintenance and may play a role in regulation of neurodegenerative diseases such as MS.

We also looked at upstream regulation of the altered proteins. There was over a two-fold change in upregulated proteins that are regulated by Peroxisome Proliferation-Activated Receptor Gamma (Pparg). Activation of Pparg pathways promotes antioxidant defence, oxidative phosphorylation, and mitochondrial function (Corona & Duchen, 2016). Agonists of the Pparg pathway have been hypothesized to ameliorate neurodegenerative disease that have underlying inflammatory or mitochondrial dysfunction components. Upstream regulation of downregulated proteins was identified as Specificity Protein 1 (Sp1), Runt-Related Transcription Factor 2 (Runx2), and Histone Acetyltransferase p300 (EP300). Upregulation of Sp1 is associated with facilitating neurodegenerative disease pathogenesis, such as Alzheimer's (Citron et al., 2008). Runx2 pathway activation has also been associated with inhibition of neurogenesis in neurodegenerative disease (Nakatsu et al., 2023). Thus, downregulated proteins regulated by Sp1 and Runx2 may play an opposing role to facilitating neurodegeneration.

We wanted to further validate the proteomic findings and selected three targets of interest: Ncam1, Nfasc, and HSP90. We chose these targets because of their roles in remyelination and MS, along with their continuity throughout our previous experiments. As aforementioned, Ncam1 plays a role in regulation of myelin production. Specifically, research shows that Ncam1 induces the survival and proliferation of OPCs leading to increased OL survival and axonal outgrowth (Palser et al., 2009). An increase in Ncam1 may be able to provide a supportive remyelinating role in neurodegenerative diseases, such as MS. Nfasc is known to play an important supportive role at the paranodal junctions at the nodes of Ranvier on axons. In MS, disruption of Nfasc localization has been shown to precede a demyelinating insult (Howell et al., 2006). Having elevated levels of this protein may counteract this disruption to provide more support at axonal junctions during demyelination. Lastly, HSP90 was found to be upregulated in serum and the CNS of animals that received repeated RIC. HSPs carry varied roles in neurodegeneration, including stress response, chaperone functions, and cellular protection (Beretta & Shala, 2022). HSP90 may serve a neuroprotective or a detrimental function in the context of MS. HSP90 inhibitors have demonstrated anti-inflammatory effects (Feinstein et al., 2008). Furthermore, antibodies reactive to HSP90 induce OPC apoptosis in vitro (Cid et al., 2004). On the other hand, HSP90 is necessary to induce protection of neuronal cells in cellular homeostasis (Calderwood et al., 2021). An increase in HSP90 due to repeated RIC may be due to the pleiotropic nature of the protein. In alignment with our proteomic data, all three targets were significantly upregulated with 14 days of repeated RIC when compared to the no intervention controls.

We then wanted to compare the transcriptomic and proteomic data sets to determine any overlap. We found six targets that were consistently upregulated and two targets that were

consistently downregulated. We also found that three gene ontology pathways were consistently upregulated: myelination, nervous system development, and brain development. In breaking down the myelination pathway, the three targets that contributed to this in both the transcriptomic and proteomic datasets were CNTNAP1, MBP, and PLEC. CNTNAP1 is a regulator of neural progenitor cells and neurodevelopment. Mutations in CNTNAP1 have been linked to autosomal recessive diseases such as Lethal Congenital Contracture Syndrome Type 7 (LCCS7) and Congenital Hypomyelinating Neuropathy Type 3 (CHN3) (Sabbagh et al., 2020). Research has shown that dysfunctional CNTNAP1 disrupts the maintenance of the paranodal junction which attenuates myelinating efficiency (Hengel et al., 2017). MBP is a highly abundant protein in the CNS. MBP is commonly known for its adhesion to the surface of myelin. MBP also functions as a membrane actin-binding protein, and an intracellular, extracellular, and nuclear signaling protein (Boggs, 2006). The presence of MBP is a crucial aspect of myelination during development, and remyelination after injury (Lindner et al., 2008). As we previously described, plectin is an abundant CNS cytolinker that also has signaling functions. Abnormal plectin disrupts the integrity of the BBB (Lie et al., 1998). Despite these many links to MS pathophysiology, plectin has not been fully explored as a potential therapeutic target or biomarker of the disease. One study notes that the *PLEC* gene to be downregulated in 14 of 19 MS tissue samples (Liu et al., 2022). To our knowledge, this is one of the only studies that links plectin with MS. If *PLEC* is downregulated in MS and low levels of plectin can exacerbate injury, RIC-induced upregulation of this target may serve a counteractive role.

We have shown that repeated remote ischemic conditioning induces a broad spectrum of transcriptomic and proteomic changes to the naïve central nervous system. We now wanted to assess whether some of these changes would be beneficial in an injury model. Specifically, we

chose the LPC-induced demyelination model of MS for mice. LPC is a nonspecific, lipid disruptor that is injected into the T3-T3 intervertebral space to induce a focal demyelinating lesion (Plemel et al., 2017). We chose this model to use with repeated RIC because it has a clear timeline of demyelination, followed by remyelination. Furthermore, this model focuses on the pathophysiology of demyelination and does not include a significant immune component. Using the core lesion slices, we compared the size of the lesion area between a sham intervention group of mice with the LPC injury and mice that received the LPC injury and 14 days of RIC. No significant changes in lesion size were found between groups. This could be explained by the very harsh nature of the LPC injury model. An intervention that is broad, such as RIC, may not have significant enough effects to counteract the focal LPC injection into the mouse CNS. However, we also wanted to assess any changes to the cellular microenvironment of the lesion with repeated RIC.

Oligodendrocyte precursor cells are important in the process of remyelination. Therefore, we sought to determine if repeated RIC induced any increase in proliferation of OPCs in the lesion area. We used a pan proliferation marker (Ki67) and oligodendrocyte lineage marker (Olig2) to highlight any OPCs that were in a proliferative state. We compared the number of double stained Olig2+/Ki67+ cells in the lesion area of LPC-injured mice that received sham or 14 days of repeated conditioning. The results showed that there was no difference in numbers of this population of cells. However, upon removing the Olig2+ quantification, the data showed that there was a significant increase in simply Ki67+ cells in the repeated RIC group compared to sham. This may indicate that there is a different cell type in the lesion environment that is being affected by this intervention. Future experiments are required in order to elucidate which cell type may display increased proliferation due to repeated RIC in the lesion.

Based on the RNA sequencing and proteomics data we obtained in aim 2, we selected a few upregulated markers to stain in the lesion that may play a role in remyelination. As previously described, both neurofascin and plectin may play roles in white matter repair and neuroprotection. We stained for these markers in the lesions of animals that received sham RIC and 14 days of repeated RIC. After quantification, in both cases neurofascin and plectin were upregulated in the lesion area of animals that received 14 days of repeated RIC. This is also consistent with our previous findings wherein neurofascin protein and plectin RNA and protein were upregulated in the uninjured CNS.

Finally, we wanted to further explore the idea that repeated RIC may induce changes that can promote white matter repair and protection. We stained lesions for a well-known myelin marker (MBP) and NFH, which is a neuronal axon marker. In comparing the sham intervention lesion to the repeated RIC lesion, MBP but not NFH was significantly upregulated in the latter group. This indicates that while the number of axons is not changing due to repeated RIC, there is an increased amount of myelin. There are some limitations to note with these experiments. Primarily, the question of whether this increase in myelin in the repeated RIC group is due to increased production of myelin or increased myelin debris. Moreover, the gold standard for evaluating remyelination is typically electron microscopy (EM), which was not used in the context of this thesis. Further experiments that use EM are required to solidify the claim that there may be more remyelination due to repeated RIC.

Chapter 5: Conclusion

The purpose of this thesis was to explore the potential for repurposing repeated remote ischemic conditioning as an intervention for white matter damage in the context of multiple sclerosis. To the best of our knowledge, RIC has not been fully explored in the context of this disease. Furthermore, *repeated* RIC compared to a single application of the intervention has also not been fully explored in the literature. We initially hypothesized that we would see broad, systemic changes with repeated remote ischemic conditioning that can be conferred to the CNS. We also expected to see upregulation of pathways and markers that are associated with attenuating the protecting against the white matter damage observed in pwMS. After developing the RIC protocol, we were able to show that single and repeated RIC altered levels of specific targets in the mouse spinal cord at the transcriptomic and proteomic levels. Some of these targets were associated with pathways involved in remyelination, axonogenesis, and neuroprotection. In an animal model of MS, we had hypothesized that we would see changes due to RIC in the lesion that could be categorized as neuroprotective. We concluded that RIC did not affect lesion size in the LPC-induced demyelination model of MS. However, we did document increased levels of markers such as neurofascin and plectin in the lesion area. We also note that there was an increased level of MBP and increased level of an unidentified proliferating cell in the repeated RIC spinal cord lesion when compared to non-intervention animals. Further research is required to investigate the questions that developed with the current study. In summary, there is promising potential for the repurposing of repeated RIC in the context of white matter repair and protection but requires further research.

Chapter 6: References

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