Novel Strategy to Protect and Induce Repair in Experimental Autoimmune Encephalomyelitis (EAE)

A Thesis Submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Department of Anatomy and Cell Biology

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that is characterized by immune-mediated segmental demyelination and variable degrees of axonal and neuronal degeneration. Efficient repair of demyelinated lesions is one of the major challenges of MS. Conventional therapeutic approaches tend to focus on modulation of the immune response responsible for the generation of these lesions. While this may help to alleviate some symptoms and mitigate damage, immune system modulation alone does not tackle the fundamental problem of remyelinating the damaged areas of the nervous system. In MS, demyelination can be followed early on by efficient remyelination, supporting that endogenous repair mechanisms exist. Our lab focuses on therapies that enhance intrinsic repair mechanisms of the injured nervous system. We find that acute intermittent hypoxia (AIH; intermittent periods of reduced oxygen), a non-invasive therapy, improves outcomes in spinal cord and peripheral nerve injured animals, the latter in a manner akin to electrical stimulation, likely due to AIH's ability to increase neural activity. But its potential for repair in MS is unknown.

We hypothesized that AIH treatment would enhance repair of the demyelinated CNS and mitigate experimental autoimmune encephalomyelitis (EAE) disease progression in the MOG_{35-55} EAE mouse model of MS. AIH or Normoxia control treatments were administered either at onset of EAE disease (score = 1) or at near peak EAE disease (score = 2.5) once daily for 7 days, with EAE mice followed for an additional 7 days post-treatment. Animals were examined daily for changes in clinical scores and spinal cord tissue at the end of clinical score analysis was processed for histopathological analysis to assess the impact that AIH treatment has on the degree of myelination, axonal integrity, oligodendrocyte precursor cell (OPC) recruitment, and immune response modulation. Analysis of clinical scores showed that 7 days of daily AIH treatment significantly improved clinical scores when treatment was started at near peak EAE disease but showed no significant changes when started at onset of disease. AIH treatment at near peak of disease, as compared to the Normoxia treatment group, resulted in significantly elevated levels of myelin basic protein (MBP), axon protective phosphorylated neurofilaments, reorganization of node of Ranvier Caspr+ve paranodes and OPC recruitment. I also observed a quicker resolution of the inflammatory response and the polarization of macrophages/microglia toward a pro-repair M2 phenotype. Collectively, these findings support a role for AIH treatment as a non-invasive therapeutic strategy to enhance CNS repair following demyelination.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIH	Acute intermittent hypoxia
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
Caspr	Contactin-associated protein
CD206	Mannose receptor
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
EAE	Experimental autoimmune encephalomyelitis
ED-1	CD68
ES	Electrical stimulation
GAP43	Growth-associated protein 43
HIF1α	Hypoxia inducible factor 1-alpha
IF	Immunofluorescence
IFN-γ	Interferon-γ
iNOS	Inducible nitric oxide synthase
LPC	Lysophosphatidyl choline
LPS	Lipopolysaccharide
LTF	Long term facilitation
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC-II	Class II major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
NAWM	Normal appearing white matter
NF	Neurofilament
NFH	Neurofilament heavy
NFL	Neurofilament light

NFM	Neurofilament medium
NO	Nitric oxide
NT-3	Neurotrophin-3
OCT	Optimal cutting temperature
OPC	Oligodendrocyte precursor cell
PBS	Phosphate buffered saline
PDGFRα	Platelet-derived growth factor receptor alpha
PLP	Proteolipid protein
pNF	Phosphorylated neurofilament
PNJ	Paranodal axoglial junction
PPMS	Primary-progressive multiple sclerosis
ROI	Region(s) of interest
RRMS	Relapsing-remitting multiple sclerosis
SEM	Standard error of the mean
SMI31	Phosphorylated neurofilament
SPMS	Secondary-progressive multiple sclerosis
TGF-β	Tumor growth factor beta
TNFα	Tumor necrosis factor alpha

CHAPTER 1: INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). MS is considered an autoimmune disease that is characterized by immune-mediated segmental demyelination and axonal/neuronal degeneration. While there were accounts of MS in earlier years, in 1868, Jean-Martin Charcot was the first to give a detailed description of MS as a distinct disease associated with specific clinical symptoms and pathologies involving both the spinal cord and brain[1]. It is estimated that in 2016 there were approximately 2.2 million cases of diagnosed MS globally[2]. As of 2014-2015, Canada reported one of the highest rates of MS in the world with a prevalence of approximately 260 individuals per 100,000[3].

1.1.1 MS Classifications

MS manifests in patients through a variety of different clinical and pathological features and as such, has multiple phenotypes due to this heterogenous nature[4]. MS relapses (also known as flare-ups, exacerbations or attacks) are defined to be the occurrence of new symptoms or the worsening of old symptoms[5]. These symptoms may manifest themselves as a number of features, including but not limited to, numbness or weakness of limbs, tremors, unsteady gait, loss of vision and fatigue[6]. MS can be subclassified in two ways, by clinical progression or by pathological progression. When defining MS phenotypes by clinical progression, the majority of MS cases are classified as relapsing-remitting MS (RRMS), in which an attack occurs (relapse) followed by the patient recovering fully or partially (remission) with symptoms resolving or ameliorating[7]. Some RRMS individuals experience a gradual decline in function and as a result, RRMS transitions into secondary progressive MS (SPMS) where remissions are rare. Yet another cohort of patients (about 10% of cases) will develop primary progressive MS (PPMS) where a steady decline in neurological function occurs from the time of disease onset[8–10].

Another way to stratify MS diagnosis stems from insights gained from pathological analyses of brain biopsy samples taken to resolve diagnosis. A key pathological feature of MS is the development of multiple lesions or plaques in the CNS. Lesions are described as focal areas of demyelination[11]. It is believed that there are four immunopatterns (patterns I, II, III and IV) of MS that show varying types of lesions (reviewed in [4]). All four patterns of active lesions show

infiltration by T cells and macrophages. In both pattern I and II, sharp demarcated perivascular lesions are observed and when differentiating between pattern I and II, it was found that pattern I, unlike pattern II, lacks immunoglobulin deposition and complement activation on T cells[4]. Pattern I myelin loss is thought to be mediated by toxic factors that are produced by activated macrophages. Pattern II shows equal myelin loss with active demyelination as well as loss of oligodendrocytes at the active border and is thought to be induced by antibody-mediated and complement-mediated mechanisms. Remyelinated shadow plaques are visible in both pattern I and II (reviewed in [12]). Pattern III is characterized by poorly defined active lesions and preferential loss of myelin-associated glycoprotein (MAG) with associated oligodendrocyte apoptosis in the areas of MAG loss which may be caused by metabolic processes such as mitochondrial dysfunction (reviewed in [12]). Lastly, pattern IV which is the most uncommon pattern (~1% of cases) is characterized by nonapoptotic oligodendrocyte death in periplaque white matter (reviewed in [4]).

Further, most conventional therapies that are commonly used in the treatment of MS tend to focus on immunomodulation. However, due to the heterogeneity between cases observed through combined clinical and pathological features (as noted above), these therapeutics affect patients differently and while one may be beneficial for someone, it may not show any effects in another person[4,13]. Studies such as those by Lucchinetti et al.[4] have exhibited this and provide data that shows that MS may have various mechanisms dependent on its pathologic subclassification. Therefore, with newly identified treatment targets as well as further diagnostic tools and pathological insight based on these differences in classification of MS, MS researchers are beginning to see a refinement in early diagnostic and therapeutic approaches.

1.1.2 MS Pathogenesis and Immune Response

Currently, the etiology and pathogenesis of MS is unclear, however, whether initiated by oligodendropathy or an autoimmune response, common underlying features remain such as segmental demyelination and axonal/neuronal degeneration. As a result, the conduction of action potentials along axons becomes poor and patients show characteristic symptoms of demyelination and neurodegeneration.

As stated above, demyelinated lesions throughout the CNS are a well-known feature of MS and all types of lesions (active, chronic, inactive) show inflammation and infiltration by

immune cells. Infiltrates that are comprised largely of lymphocytes and macrophages are also variable depending on their stage of demyelination[4]. T lymphocytes (T cells) are believed to be the primary immune cell mediators of MS and are predominantly found in active lesions. B cells are also found along with T cells within active lesions but to a lesser degree[14].

Magnetic resonance imaging (MRI) studies have also shown that active lesions demonstrate disruption and leakiness of the blood brain barrier (BBB) which leads to transendothelial migration of activated leukocytes[15,16]. Disruption of the BBB may be due to the increased number of proinflammatory cytokines and the generation of oxygen and nitrogen free radicals during inflammatory events within active, demyelinating lesions[17]. Another pathological feature of MS is the increased levels of antibodies in a patient's CNS. This is visualized as higher levels of oligoclonal bands, which are bands of immunoglobulins, in the cerebrospinal fluid of a patient[18,19].

Because MS is a complex disease with multiple phenotypes due to its heterogenous nature, insights into the immune, myelin and neurodegenerative states define the goals of therapeutic approaches aimed either at preventing or mitigating attacks with strategies to effect/enhance the intrinsic ability of the nervous system to repair itself.

1.2 Central Nervous System Structure and Function

The CNS is the part of the nervous system that consists of the brain and spinal cord. It is responsible for receiving information from the entire body, integrating that information and responding accurately, in a coordinated fashion. Neurons, which are defined as highly specialized cells that transmit nerve impulses, are a primary component of the nervous system. The basic anatomical structure of a neuron consists of dendrites, a cell body (also known as the soma), an axon and an axon terminal. Dendrites are branched projections that extend from the cell body of a neuron. They receive impulses from other neurons at synapses that are then transmitted to the cell body. The cell body contains the axon hillock which is the location at which incoming signals from synaptic inputs are summated and integrated before being transmitted to the axon. The axon is responsible for propagating the impulses to axon terminals which then synapse onto other neurons. Axons are surrounded by the myelin sheath and have gaps in myelin called the nodes of Ranvier[20]. There are two main types of tissues in the CNS: white matter and gray matter. With respect to neurons, white matter consists mainly of axons while gray matter consists of nerve cell

bodies, dendrites and axons[21]. Both tissue types also contain glial cells that support and protect neurons[22]. Three main glial cells in the CNS include astrocytes, oligodendrocytes and microglia. Astrocytes provide a connection between neurons and vasculature which allows for nutrients and other substances to reach the neurons. Oligodendrocytes are the main cells of the CNS that generate and maintain the myelin sheath that surrounds axons[20]. Lastly, microglia are immune effector cells of the CNS and will respond to injury/pathogens with phagocytosis[23].

While recent literature has expanded the role for astrocytes in MS beyond that of only forming a central scar following demyelination to include lymphocyte recruitment, alterations in BBB integrity allowing influx of peripheral immune cells and promotion of repair (reviewed in [24]), the major foci of MS research remain on elucidating the impact of MS on the myelin sheath, oligodendrocyte function and differentiation, axonal and neuronal degeneration and immune cell modulation.

1.2.1 Myelin Sheath

The myelin sheath is a modified plasma membrane that wraps around axons of nerves. Myelin facilitates rapid saltatory conduction of nerve impulses within axons. It was first defined by Rudolf Virchow in 1854 and originally believed to be secreted by neurons[25]. However, it is known now that the myelin sheath of the CNS is actually produced and maintained by oligodendrocytes.

1.2.1.1 Myelin Structure

Myelin wraps around axons in multiple, concentric layers to produce a uniformly thick membrane surrounding the axon. Using electron microscopy, it was visualized that compacted myelin forms a structure alternating light (intraperiod line) and dark (major dense line) layers. The intraperiod line consists of apposed outer membranes while the major dense line represents condensed cytoplasmic surfaces. Typically, the length of myelinated segments of the axons are about 150μm[26]. The paranodal domain is found on the edge of the myelin sheath segment and the juxtaparanode is directly adjacent to it. Between the edges of two myelinated segments (or paranodes), are breaks in the myelin sheath containing the nodal regions which are called the nodes of Ranvier. Paranodal axoglial junctions (PNJ) are found at the edges of each myelin

segment and they attach paranodal loops of the myelin sheath to the axon[27]. These tight junctions prevent the loss of current and allow for fast nerve conduction[26–28].

1.2.1.2 Myelin Composition and Nodal Regions

Myelin is composed of a variety of proteins and lipids. The specific proportions of both of these components allow the myelin sheath to produce and maintain its efficient insulating properties. Relative to other plasma membranes, the myelin sheath has a high lipid content (70-75%)[29]. Lipid classes found within the myelin sheath include saturated, long-chain fatty acids, glycosphingolipids (~20%) and cholesterol (~40%). Myelin thickness is in large part thought to be determined by the levels of saturated, long-chain fatty acids. Differing areas of a myelin segment contain differing distributions of proteins. Two of the most predominant proteins in CNS myelin are myelin basic protein (MBP) and proteolipid protein (PLP). Within the major dense line, MBP stabilizes the compaction of adjacent cytoplasmic membrane surfaces. One of the most abundant transmembrane proteins is PLP. While its function is not fully understood, it has been speculated that PLP is involved in the tight apposition of membrane sheaths. To provide effective electrical insulation, PNJs form a barrier between nodes and the part of the axon titled the internodal region[30]. PNJs also separate voltage-gated sodium channels found at the nodes from the juxtaparanodal potassium channels. PNJs contain cell adhesion molecule complexes, constituting of contactin-associated protein (Caspr), contactin-1 and neurofascin. Further, the internodal region contains myelin-associated glycoprotein (MAG) that is sequestered around the perimeter of the adaxonal membrane. MAG is believed to trigger downstream signaling cascades which may stimulate the maturation of the axonal cytoskeleton and thickening of the axon. Myelin oligodendrocyte glycoprotein (MOG) is another transmembrane glycoprotein that is located on the surface of myelin and oligodendrocytes. MOG is thought to be involved in the transmission of extracellular information to the inside of oligodendrocytes. MOG has also been implicated in demyelinating diseases of the CNS, i.e. experimental autoimmune encephalomyelitis (EAE), as a target antigen within autoimmune aspects of disease[31].

The gaps in the myelin sheath/nodes of Ranvier are highly specialized and essential for rapid saltatory conduction that allows for efficient propagation of impulses along an axon[32]. Voltage-gated sodium channels are concentrated at the nodes of Ranvier and are responsible for depolarization[33,34]. In response to the initiation of CNS myelination, nodal proteins and ion

channels cluster forming the nodes of Ranvier[35,36]. Further, mature nodes are able to be distinguished by the presence of Caspr along the border of the paranodal junction which are visible at later stages of myelination[37].

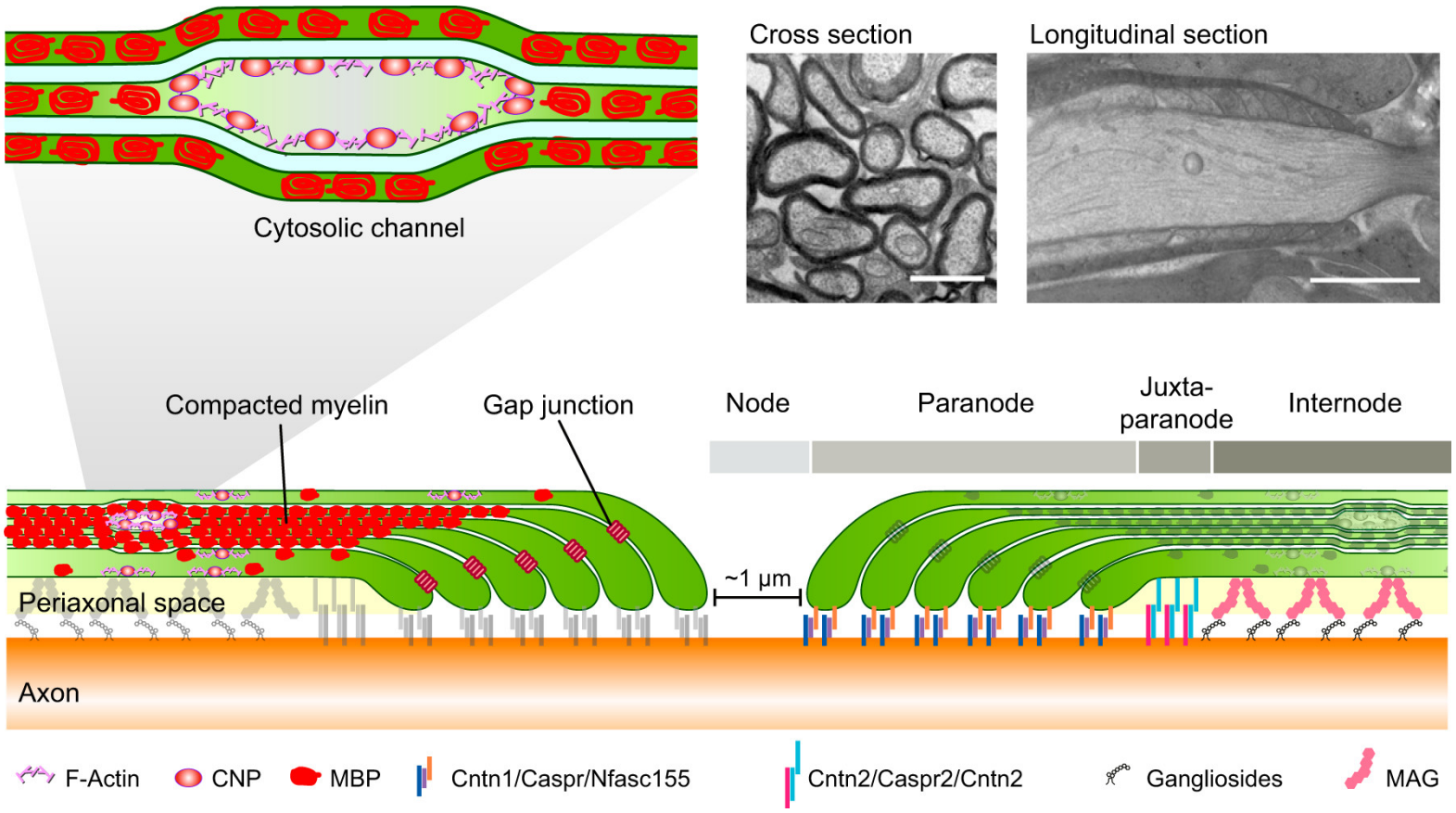

Figure 1. Schematic illustration of central nervous system myelin structure. Graphical illustration of myelin structure including the different domains of myelinated axons. Myelin basic protein (MBP) is essential in generating compacted myelin membrane stacks by zippering the cytoplasmic surfaces closely together. 2′,3′-Cyclic nucleotide 3′-phosphodiesterase (CNP) interacts with the actin cytoskeleton and counteracts the polymerizing forces of MBP, thereby generating cytoplasmic channels within the myelin sheath. Gap junctions connect the paranodal loops of myelin at the lateral edges of myelin. MAG, myelin-associated glycoprotein. Taken with permission from [28].

1.2.2 Oligodendrocytes

The primary function of an oligodendrocyte is to generate the myelin sheath around axons in the CNS[28]. A single oligodendrocyte can myelinate up to 50 axons and thus, has the ability to impact the physiology of multiple axons, unlike their peripheral nervous system counterpart, the Schwann cell[38]. Another distinguishing feature from Schwann cells is the requirement to generate new oligodendrocytes following a demyelinating insult. Therefore, new mature oligodendrocytes must be derived from oligodendrocyte precursor cells (OPCs) in order to either myelinate de novo or remyelinate axons following a pathological demyelinating event. The ability to histopathologically distinguish OPCs from oligodendrocytes is aided by the expression of selective factors. OPCs can be identified by the expression of NG2 chondroitin sulfate proteoglycan and platelet-derived growth factor receptor alpha (PDGFRα), while the commonly used transcription factor, Olig2 is not as selective as it identifies both OPCs and some mature oligodendrocytes[39,40].

While a large portion of OPCs differentiate into mature oligodendrocytes, a small subset remains undifferentiated[41]. An important function of these undifferentiated OPCs within the CNS is responding to injury and disease by being recruited to regions needing myelin repair and differentiating into mature oligodendrocytes that will hopefully repair the damaged myelin[39]. Because of this important role, failure of OPC proliferation, migration to the area needing repair and differentiation (known as oligodendropathy) may prevent the remyelination of axons. If the oligodendrocyte is metabolically compromised then the loss of metabolic support to neurons from oligodendrocytes can further advance axonal degeneration[42]. There is evidence that shows how reduced levels of OPCs and oligodendrocytes in MS lesions may lead to reduced remyelination[43–45]. While MS has been largely thought to be an autoimmune disease, recent studies have shown that myelin dysfunction, likely due to oligodendropathy, may lead to further axonal damage and degeneration[46].

Potential treatment strategies for oligodendropathy-related demyelination and degeneration in a neurodegenerative disease such as MS include either protecting oligodendrocytes from apoptosis or strategies that enhance proliferation and recruitment of OPCs to areas of inflammation and demyelination and improve differentiation to oligodendrocytes within those regions. With the enhancement of oligodendrocyte function, improved metabolic support and remyelination may be possible. Multiple inducers of OPC proliferation and

differentiation have been identified. For example, macrophages/microglia that are polarized toward an anti-inflammatory (M2) phenotype secrete activin-A. Activin-A has been found to enhance the differentiation of OPCs to oligodendrocytes[47]. Similarly, neurotrophin brain-derived neurotrophic factor (BDNF) has been shown to promote the formation of new oligodendrocytes in the injured spinal cord[48]. Both of these OPC/oligodendrocyte proliferation and differentiation inducers are potential therapeutic targets for enhancing remyelination and regeneration.

1.2.3 Axons

Demyelination in MS is not without consequences on axonal integrity and dysfunction. Axons are long projections that extend from the cell body of a neuron. Their main function is to conduct electrical impulses away from the nerve cell body, but they are also important for the transport of signals associated with neuronal function and survival[49]. With respect to neurodegenerative diseases like MS, biochemical changes in cytoskeletal proteins, in particular the intermediate filaments, is one of the factors that can be readily identified and is also linked to susceptibility associated with axonal degeneration[50]. Additional cellular events linked to an increased susceptibility of axons to degeneration in demyelinating disorders include mitochondrial dysfunction and disrupted axon transport of organelles (i.e. mitochondria) to distal portions of the axon[51,52]. Evidence has also come forward supporting that axonal degeneration is not only linked to demyelinating events in MS, but may also be causally linked to driving the pathology as axon loss has also been observed in regions of MS biopsies that are otherwise "normal appearing white matter" (NAWM)[53]. Whether the axonopathy precedes or is a consequence of demyelination is an ongoing debate and has led to the development of two models – the outside-in model and the inside-out model of axonal degeneration as driving much of MS pathogenesis and disability (Figure 2; reviewed in [54,55]). Resolution of this debate will help in the development of therapeutic approaches.

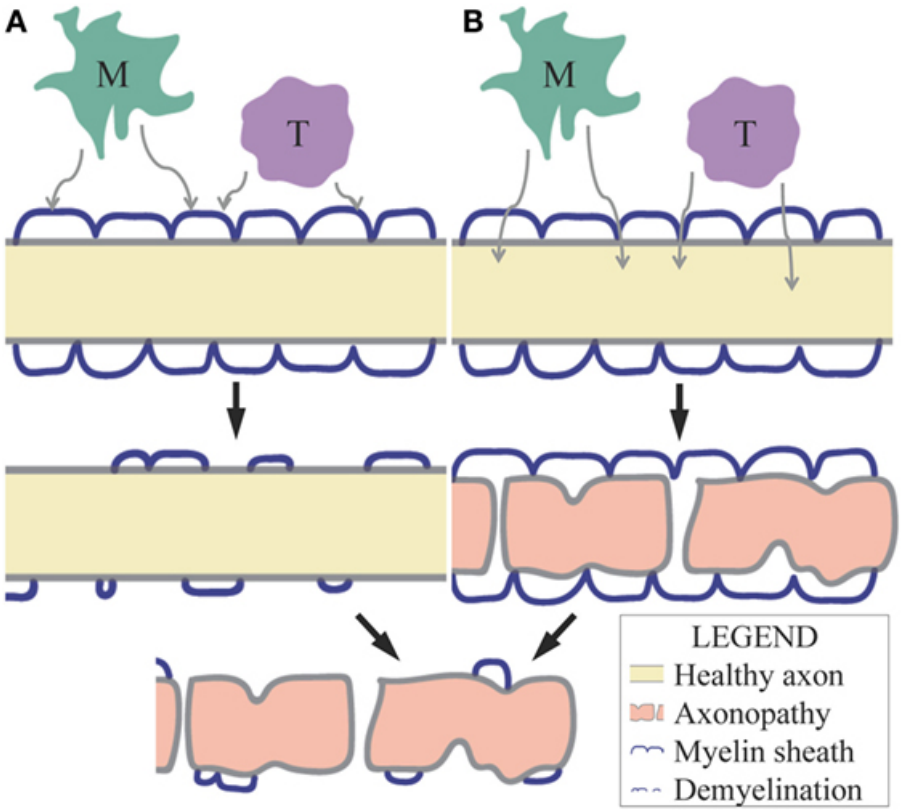

Figure 2. Schematic illustration of outside-in vs inside-out models of axonal degeneration. **(A)** The outside-in model of axonal degeneration predicts that demyelination occurs before axonopathy due to myelin-degenerative factors released by inflammatory cells (M = macrophages/microglia, T = T lymphocytes) within the CNS. The loss in protection and trophic support by myelin results in axonal damage in this model. **(B)** The inside-out model of axonal degeneration or Wallerian degeneration predicts that axonopathy occurs before demyelination due to neurotoxic factors released by inflammatory cells within the CNS. These factors may diffuse through myelin or pass through the nodes of Ranvier. Taken with permission from [54].

1.2.3.1 Neurofilaments and Axon Susceptibility to Degeneration

Neurofilaments (NF) are intermediate filaments that are abundant inside axons, with alterations in their biochemistry linked to degenerative events in axons in MS. They provide structural support to axons and are necessary for regulating axon diameter and transmission of nerve impulses[56,57]. Within the CNS, NF are made up of four subunits: NF light (NFL), NF medium (NFM) and NF heavy (NFH) polypeptides as well as α-internexin[58]. NFM and NFH can be post-translationally modified through phosphorylation[59]. Phosphorylation is regulated by protein kinases and phosphatases and occurs at the lysine-serine-proline (KSP) sites of the C-terminal domain of NFM and NFH within myelinated axons[60]. It has been shown that phosphorylation of NF is dependent on the myelination states of the axon, with decreased NF phosphorylation in hypomyelinated or unmyelinated regions of axons[61–63]. Dephosphorylation of NF increases the susceptibility of the NF to proteolysis by calcium-activated neutral proteinase calpain[64]. Studies have suggested that a possible function of the phosphorylation of NF is to protect and resist the degradation linked with calpain[64,65]. In MS, the phosphorylation state of NFs is linked to axon pathology and demyelination, with hypophosphorylation and aberrant hyperphosphorylated regions linked to the axonal pathology and the former – i.e. reduction in phosphorylated NF (identified with SMI31 antibodies against phosphorylated NFs) associated with white matter lesions[66,67]. More recently, a pilot study has shown that higher serum levels of phosphorylated NFH coupled with inflammatory cytokines might serve as biomarkers of disability and axonal damage in RRMS[68].

1.2.4 Macrophages/Microglia

MS lesions are typically staged by the presence and density of macrophages/microglia observed in pathological samples of CNS tissue[67]. Monocyte-derived macrophages and their CNS resident counterparts, microglia, are important effector cells of the immune system and contribute to both homeostasis and disease in the body[69]. They are involved in both the innate immune system as well as the adaptive immune system. In the innate immune system, they are phagocytic cells that are able to clear debris and pathogens from the body[70]. Activated macrophages are also able to secrete cytokines and chemokines that contribute to the immune response. Both of these components are involved with the initiation of inflammation[71]. Macrophages are antigen-presenting cells and are capable of activating the adaptive immune system. This is done through

the presentation of the material that the macrophage engulfed during phagocytosis (also called antigen) on its cell surface through a class II major histocompatibility complex (MHC-II). This presentation of antigen signals the pathogen/foreign substance to T-helper cells. T-helper cells are then able to signal for and activate other cells of the adaptive immune system, such as cytotoxic T cells, to remove the pathogens. They also stimulate B cells to secrete antibodies to that specific antigen[72].

1.2.4.1 Macrophage Phenotypes and Disease State

Macrophages are differentiated from monocytes derived from stem cells in the bone marrow that leave the blood and enter the tissue. Further, monocyte subsets have environmental and phenotypic differences which affect the differentiation and functioning of macrophages[73]. Currently, macrophages are thought to differentiate across a large spectrum of activation ranging from classically activated "M1" macrophages on one end to alternatively activated "M2" macrophages on the other[74]. M1 macrophages are activated during cell-mediated immune responses through exposure to molecules such as lipopolysaccharide (LPS) or the T_H1 lymphocyte product, interferon-γ (IFN-γ)[74,75]. These M1 phenotype macrophages show high levels of phagocytotic activity and produce and secrete proinflammatory cytokines (i.e. tumor necrosis factor alpha (TNFα)), nitric oxide (NO) and reactive oxygen intermediates, which led to their subsequent designation as "proinflammatory" macrophages[75–77]. Comparatively, M2 macrophages are activated through exposure to T_H2 responses including certain anti-inflammatory cytokines such as IL-4, IL-10, IL-13 or tumor growth factor beta (TGF-β)[76,78]. M2 macrophages express mannose receptor, CD206, which further promotes the expression of anti-inflammatory molecules, and activin-A, which as mentioned earlier can promote the differentiation of OPCs into mature oligodendrocytes, a critical process in remyelination[47,77]. The variations in expression of molecules seen in proinflammatory M1 macrophages versus anti-inflammatory M2 macrophages allow us to use these distinct markers to distinguish between the two opposing ends of the macrophage polarization continuum. Further, as a result of the differences between M1- and M2-type macrophages, observable differences in function also exist, particularly in CNS injury.

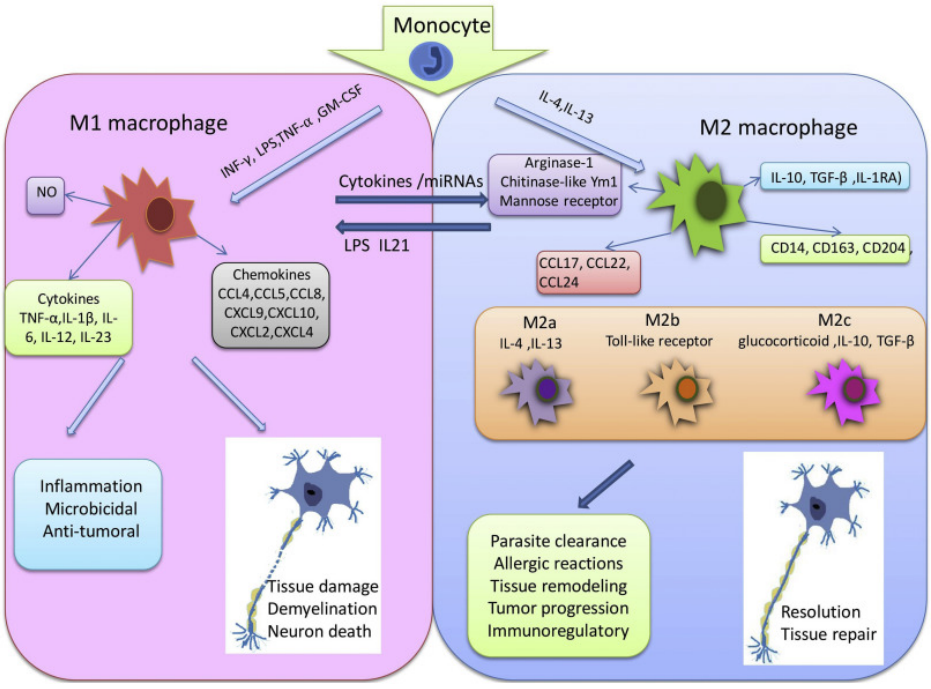

Figure 3. M1/M2 polarization of macrophages and their functions. The M1 phenotype of macrophages (proinflammatory) is induced by interferon (INFγ), lipopolysaccharide (LPS), tumor necrosis factor (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF). They can produce proinflammatory cytokines, chemokines, and nitric oxide (NO). Moreover, they show an increased ability to present antigens, cause phagocytic infections induced by bacterial, viral, or fungal factors, as well as kill tumor cells. During the early phases of MS and EAE, they are able to induce tissue damage, demyelination, and neuronal death in the inflamed CNS. M2 phenotype macrophages are induced by interleukin IL-4 and IL-13, and they can produce IL-10, transforming growth factor (TGFβ), and IL-1 receptor antagonist (IL-1RA) as well as show high expression of markers of alternative activation (i.e., arginase-1, chitinase-like Ym1, mannose receptor, and the receptors CD14, CD163, CD204, CCL17, CCL22, and CCL24). Unlike M1 macrophages, they do not display any cytotoxic properties, they contribute to parasite clearance, and, in allergic reactions, they have functions related to tissue remodeling, angiogenesis promotion, tumor progression, and immunoregulation. During the later phases of MS and EAE, they are responsible for inflammation resolution and tissue repair. M2-polarized macrophages are further subdivided into M2a (elicited by IL-4 or IL-13), M2b (following stimulation by immune complexes in the presence of a Toll-like receptor ligand), and M2c (when exposed to anti-inflammatory stimuli such as glucocorticoid hormones, IL-10, or TGF-β). Taken with permission from [79].

1.2.4.2 Macrophages and CNS Injury/Repair

Macrophages/microglia play an important role during CNS inflammatory events. In response to CNS injury, macrophages and microglia react rapidly by invading regions of damage with a primary function of phagocytosis of myelin debris[80]. Fast debris clean up results in a better chance of remyelination and repair. Activated macrophages have also been shown to release neurotrophins such as neurotrophin-3 (NT-3) and BDNF which are known to be involved in nervous system regeneration[81]. However, while carrying such an important function, macrophages/microglia are also known to contribute to pathology through the presentation of antigens to cytotoxic T cells and the release of toxins and inflammatory cytokines[82,83]. As stated above, they are highly plastic and are able to switch between phenotypes depending on the environment. Unfortunately, poor balance between phenotypes can lead to pathogenesis, injury and disease[84]. M1 macrophages are generally involved in the induction of the acute phase of inflammation and are associated with bactericidal and antimicrobial mechanisms that lead to pathogen death followed by debris phagocytosis within early "healing" stages[76,85]. On the other side, M2 macrophages are associated with homeostasis and the switch from M1 to M2 phenotype that coincides with the initiation of repair processes such as remyelination through the promotion of OPC differentiation in an activin-A dependent manner and clearance of apoptotic cells[47,76,78,86]. In the EAE animal model of MS, elevation in M1 polarized macrophages is observed during peak EAE disease and the suppression of M2 polarized macrophages leads to worse clinical disease scores[87,88]. Therefore, treatment approaches that enhance macrophage polarization from M1 to M2 may be suitable to aid in repair and remyelination of CNS injury.

1.3 Injury, Dysfunction and Repair in Multiple Sclerosis

The CNS can be affected by a wide array of diseases including metabolic disorders, degenerative conditions, traumatic injuries, etc[89]. This thesis focuses directly on the damage exhibited by the demyelinating neurodegenerative disease, MS, and the potential for enhancing intrinsic repair mechanisms.

1.3.1 Demyelination and Neurodegeneration in Multiple Sclerosis

Demyelination is described as damage to, or loss of the myelin sheath around nerves of the nervous system. It may be caused by a number of problems such as inflammation, infection,

viruses or disease. Demyelination mechanisms in MS can be simplified into two categories: demyelination caused by inflammation (outside-in) or demyelination caused by an oligodendropathy (inside-out)[46]. With MS originally being thought of as a primarily autoimmune disease, it was believed that demyelination was largely triggered by autoreactive CD4+ T cells, similar to EAE. In this case, T cells recognize myelin antigen and initiate an inflammatory response that leads to damage[90]. However, there has been further research that shows that CD8+ T cells and B cells also have roles in initiating demyelination in MS[91,92].

Additionally, it is well known that the innate immune system has a role in MS demyelination. Innate immune cells such as microglia are able to trigger demyelination through various mechanisms such as damaging the BBB, activating T cells, inducing apoptosis, breaking down myelin, etc…[93]. Some additional factors associated with macrophage activation including cytokines, TNFα and IFN-γ, and certain antibodies against myelin antigens are elevated in both MS patients and EAE animal lesions and are believed to perhaps be involved in the progression of demyelination[94,95]. Immune-mediated and autoimmune oligodendrocyte dysfunction and death when antigens or antibodies that are expressed in oligodendrocytes are targeted can also lead to myelin damage[96,97]. Additionally, there is evidence that suggests that oligodendrocyte death in MS lesions can occur prior to actions taken by immune cells[98,99]. This suggests that primary oligodendropathy is another source of myelin damage, separate to that of damage to oligodendrocytes caused by inflammation.

Studies have also shown that axonal damage can occur and progress as a consequence of active demyelination in both MS and the EAE animal model of MS[100,101]. Axonal damage has been correlated with neurological disability in MS patients[102]. Post-mortem MS tissue analysis indicates that axonal loss also occurs in tissue that is not subject to demyelination[103]. Thus, while neurodegeneration and axonal dysfunction have been previously known to be a result of demyelination in MS, in recent years, it has also been shown to occur independent of demyelination[104]. Further, even though axonal damage may occur independent from demyelination, it is still directly associated with inflammation[14,100].

1.3.2 Factors or Indicators of Efficient Repair and Remyelination in Multiple Sclerosis

One of the major challenges of MS is the efficient repair and remyelination of demyelinated lesions. There are multiple factors that are involved in the remyelination and repair

of the CNS following an MS attack. While the nervous system can intrinsically remyelinate and regenerate to an extent, the level of repair is less than ideal. Thus, therapeutic targets that can improve intrinsic repair mechanisms are extremely valuable for CNS demyelinating diseases like MS.

The rapid and effective clearance of myelin debris is an important component for improved remyelination. The presence of myelin and myelin debris prevents effective remyelination and axon regeneration as it inhibits the differentiation of OPCs to mature oligodendrocytes. Oligodendrocytes are responsible for remyelination in MS and support axon integrity so failure of OPCs to differentiate is detrimental[105,106].

As previously stated, macrophages/microglia have an important role in both demyelination and remyelination[107,108]. While macrophages promote cytotoxicity through presentation of antigens to T cells and the release of toxins and cytokines, they are also known to phagocytose myelin debris[54,56,57]. Macrophages/microglia are also able to switch between phenotypes. In particular, a switch from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype can lead to improved repair and remyelination[51,53]. There is evidence that implicates M1-derived proinflammatory mediators and cytokines with OPC differentiation failure[109]. On the contrary, M2-derived molecule activin-A is essential for OPC differentiation into mature oligodendrocytes that can then effect efficient remyelination[47].

Further, increased neural activity in the CNS is also known to promote remyelination through the release of glutamate and growth factors[110–112]. Increased glutamate leads to enhanced proliferation and differentiation of OPCs to mature oligodendrocytes that go on to promote remyelination and repair[113,114]. There are multiple ways in which nervous system neural activity can be increased and affect remyelination and repair processes, including through voluntary exercise and electrical stimulation (ES)[115–118].

Additionally, plasticity-associated proteins are able to influence remyelination and repair through their upregulated gene expression by increased neural activity. Neurotrophins such as BDNF enhance OPC differentiation into mature oligodendrocytes and induce better remyelination[48,86]. BDNF is found distributed in both neurons and glia[119]. The loss of BDNF and dysregulation of its secretion has been linked to impaired remyelination and regeneration in nervous system disease and pathology[120,121]. Beyond increased neural activity, nervous system injury also causes an increase in the expression of BDNF[119]. While BDNF contributes to

developmental myelination and remyelination following nervous system injury, it is not known whether BDNF continues to influence motor neurons in the developed nervous system[119,122]. This indicates that BDNF has a particularly important role in the repair of pathologies. There is evidence that BDNF expression and secretion is activity dependent (reviewed in [123]). As such, therapeutic strategies such as ES are potential inducers of BDNF expression and promoters of the conversion of precursor proBDNF to its mature form[124]. Additionally, if proBDNF does not convert to its mature form, it may inhibit the infiltration of macrophages to injured areas which can lead to detrimental effects that block remyelination and repair[125]. BDNF is also implicated in having a role in the phosphorylation of neurofilaments[126]. As detailed previously, phosphorylation is linked to the protection of axons by enhancing axon resistance to calpain degradation[64].

While another plasticity marker, growth-associated protein 43 (GAP43) is expressed at very low levels in uninjured motor neurons, it is found extensively in the neuropil, primarily on endosome-like structures near the plasma membrane[127,128]. GAP43 expression is known to be involved with axon growth[129]. Similar to BDNF, GAP43 is expressed at high levels during development and during peripheral nervous system regeneration[130]. In post-mortem MS tissue, elevated levels of GAP43 are observed in remyelinated regions correlating with axon regeneration and repair[131]. It has also been shown that GAP43 expression and transport can be increased in response to CNS injury and its upregulation is linked to regeneration potential[130,132]. As a result, GAP43 has been utilized extensively as an indicator of a heightened plastic state in both CNS and PNS pathologies.

Lastly, transcription factor hypoxia-inducible factor 1-alpha (HIF1α) is another plasticity and regeneration-associated gene whose expression is affected by oxygen levels. During normoxic conditions (atmospheric oxygen levels), HIF1α is expressed at low levels due to degradation by the ubiquitin-proteasome system[133]. However, HIF1α accumulates rapidly under low oxygen level conditions (hypoxia)[134]. Increased HIF1α expression in both neuronal and non-neuronal cells has been linked to regenerative capabilities following peripheral nervous system injury and spinal cord injury[135,136].

Therefore, therapeutic strategies that alter levels of these plasticity associated genes to align with the benefits seen during intrinsic repair processes may be advantageous for the

enhancement of remyelination and repair following CNS injury due to a demyelinating disease such as MS.

1.4 Experimental Autoimmune Encephalomyelitis as a Preclinical MS Model

Preclinical models that accurately align with the pathogenesis of MS are invaluable for performing initial assessments of novel therapeutic interventions. EAE is one of the most common animal models of MS as it displays numerous features similar to the human disease. Research using the EAE model began in the early 1900s when researchers injected monkeys with emulsions or extracts from rabbit brains. These injections led to pronounced pathological changes and demyelination in the brains of the monkeys[137,138]. The use of an antigen-containing mineral oil-based adjuvant by Jules Freund called complete Freund's adjuvant was then combined with the brain emulsions/extracts to accelerate disease onset. Since these initial experiments in monkeys, the EAE model has been induced in many other animals, including mice, rats, rabbits, guinea pigs, etc. However, researchers frequently choose to use rats and mice as they are easier to obtain and care for, with the latter also amenable to genetic manipulation.

EAE is primarily an autoimmune disease mediated by inflammatory events. Production of the pathogenic neuroinflammatory response observed in EAE is caused by mononuclear inflammatory infiltration through direct and indirect mechanisms by primarily CD4+ T cells which lead to the production of myelin antibodies[139]. Demyelination and axonal damage then occur through multiple mechanisms that involve inflammatory cytokines, chemokines, macrophages and other inflammatory cells. While EAE pathology is primarily driven by CD4+ T cells, EAE models that address the involvement of CD8+ T cells have also been created that involve the adoptive transfer of these cells[140]. Similarly, assessment of the role of B cells has been addressed through the creation of a spontaneous B cell dependent model of EAE[141].

Many different EAE models are now available to researchers. This is useful in regard to MS research as no single model is ever adequate to fully describe MS and numerous models allow researchers to look at multiple aspects of the disease pathogenesis. Depending on the animal species being used in experiments, researchers use different brain proteins, including MOG, MBP and PLP, to induce EAE for best efficacy.

Both EAE and MS are characterized by immune cell infiltration of the CNS, demyelination and axonal damage. However, while EAE is predominantly a CD4+ T cell

autoimmune disease, MS also tends to involve a greater number of CD8+ T cells and B cells[142]. The consequence of this difference in disease mediation is that EAE reproduces some aspects of clinical MS but it itself is not MS and this limitation should be considered when attempting to translate findings and therapies from EAE to MS. Even so, EAE is one of the closest animal models to MS that researchers can utilize when studying disease mechanisms of and potential therapeutics for MS. The most commonly used EAE model is the MOG_{35-55} model which when induced in the C57BL/6 mouse, takes on an acute progressive pathophysiology. This model is considered to be one of the better, more robust and reproducible neuroimmunological models and, if used appropriately, is suitable to study the efficacy of therapeutic interventions and ability to mitigate or reverse disease states[143].

1.5 Acute Intermittent Hypoxia as a Novel Therapy to Promote Nervous System Repair

Intermittent hypoxia is an intervention defined as the exposure of a subject (rat, mouse, human, etc.) to hypoxia (<21% O_2) alternating with normal oxygen levels (normoxia; 21% O_2). Conventionally, hypoxia was negatively associated with pathologies such as cerebral ischemia, myocardial ischemia and tumor angiogenesis[144]. However, recently it has been shown, in both animal and human studies, to also have potentially beneficial effects in the treatment of COPD[145], in improving aerobic performance capacity and inducing altitude acclimation[146,147], in preserving ventilatory capacity in amyotrophic lateral sclerosis patients[148] and in slowing the rate of Parkinson's disease development. Two commonly used types of intermittent hypoxia exist: severe/chronic and acute. Severe/chronic intermittent hypoxia involves very low oxygen levels (2-8% O_2) and high numbers of episodes per day (48 - 2,400 episodes/day) whereas acute intermittent hypoxia (AIH) uses modest hypoxia (9-16% O_2) and lower numbers of episodes per day (3-15 episodes/day)[149].

Further, intermittent hypoxia, specifically AIH, has been identified to have the potential to induce plasticity and enhance motor function in spinal cord injuries (SCI)[150–155]. Protocols for intermittent hypoxia vary, however a recent review found that severe/chronic models of intermittent hypoxia are usually pathogenic while AIH tends to be safe and shows beneficial effects[149]. They noted that beneficial effects seem to arise when hypoxia is modest (9-16% O_2) and there are low numbers of cycles in the protocol (3-15 episodes/day). Thus, AIH holds therapeutic potential as a simple, safe and effective therapy for multiple clinical disorders.

AIH consists of brief repetitive exposures to low oxygen levels for short periods of time in alteration with normal oxygen levels. Specific levels of hypoxia and the number of cycles associated with it can induce a type of respiratory plasticity called long-term facilitation (LTF). Respiratory LTF manifests as an increase in phrenic output following hypoxic episodes. There are multiple cellular pathways and mechanisms that have been discovered that are involved in the induction of LTF following AIH. Primarily, the activation and maintenance of LTF requires the activation of serotonin receptors and subsequent serotonin-dependent synthesis of BDNF[156,157]. BDNF is associated with regeneration and remyelination in the CNS[119]. Further, AIH has been observed to increase neural activity with enhanced motor neuron firing and synaptic strength in the phrenic nerve up to 2 hours following treatment[158]. It must be noted that continuous exposure to hypoxia cannot induce LTF as it is pattern dependent and must be alternated with normal oxygen levels to produce desired plasticity effects[159]. The increases in neural activity and BDNF expression due to AIH treatment seem similar to that seen with brief ES which also imposes low levels of beneficial stress[160]. Given that we and others have shown that strategies that increase neuronal activity can be beneficial for myelin repair, immune cell modulation toward a pro-repair phenotype and axon protective phenotype, non-invasive AIH treatment may be a possible therapeutic strategy for enhancing repair and remyelination in a CNS injury similar to ES[113,117,118,161].

1.6 Hypotheses and Specific Aims

Efficient repair of demyelinated lesions is one of the major challenges of MS. Current therapies attempt to modulate immune responses that are generally responsible for demyelinated lesions. While this may be valuable, it does not solve the problem of remyelinating damaged areas of the nervous system. Therefore, our lab focuses on therapies that have the potential to enhance intrinsic repair mechanisms of the injured nervous system.

We have shown that ES has a dramatic impact on repair of lysophosphatidyl choline (LPC)-induced focally demyelinated rat peripheral nerve and spinal cord. We find that AIH, a non-invasive therapy, improves outcomes in spinal cord and peripheral nerve injured animals in a manner similar to that observed with ES, likely due to AIH's ability to increase neuronal activity and BDNF synthesis. However, its potential for repair in MS is unknown.

Based on the above, it is hypothesized that AIH treatment enhances repair of demyelinated CNS and slows EAE disease progression.

The specific aims of this research include:

1) To establish the EAE mouse model and its clinical score assessment
2) To use the EAE model to examine the effect of AIH therapy on clinical score outcomes, including:
    a. EAE disease progression
3) To use the EAE model to examine the effect of AIH therapy on enhancing intrinsic repair processes, including:
    a. Axon protection
    b. Node of Ranvier organization
    c. Oligodendrocyte precursor cell (OPC) recruitment
    d. Myelination
    e. Inflammation
    f. Immune cell polarization

CHAPTER 2: MATERIALS AND METHODOLOGY

All animal procedures were approved by the University of Saskatchewan Animal Research Ethics Board and adhered to Canadian Council on Animal Care (CCAC) guidelines.

2.1 Experimental autoimmune encephalomyelitis (EAE)

2.1.1 Experimental Animals

Female C57BL/6 mice aged 10-12 weeks (Charles River Laboratories; MA, USA) were used for the following experiments (total n = 85). All animals were housed 3-5 mice/cage under a 12 hour light:dark cycle, with standard chow and water, at the Lab Animal Services Unit (LASU) at the University of Saskatchewan. All animals were acclimated for 7 days prior to any experiments/procedures.

2.1.2 EAE Induction

EAE was induced using the Hooke Laboratories EK-2110 EAE kit containing MOG_{35-55}, complete Freund's adjuvant and pertussis toxin (Figure 4)[162]. On day 0, female C57BL/6 mice (Charles River Laboratories) were immunized subcutaneously at two sites (upper back between front limbs and lower back between hind limbs), 0.1 mL/site, on the dorsal side for a total of 200μg MOG_{35-55} in complete Freund's adjuvant. The mice were also injected intraperitoneally with pertussis toxin solution (pertussis toxin diluted in phosphate buffered saline (PBS)), 0.1 mL/dose, on day 0 and day 1.

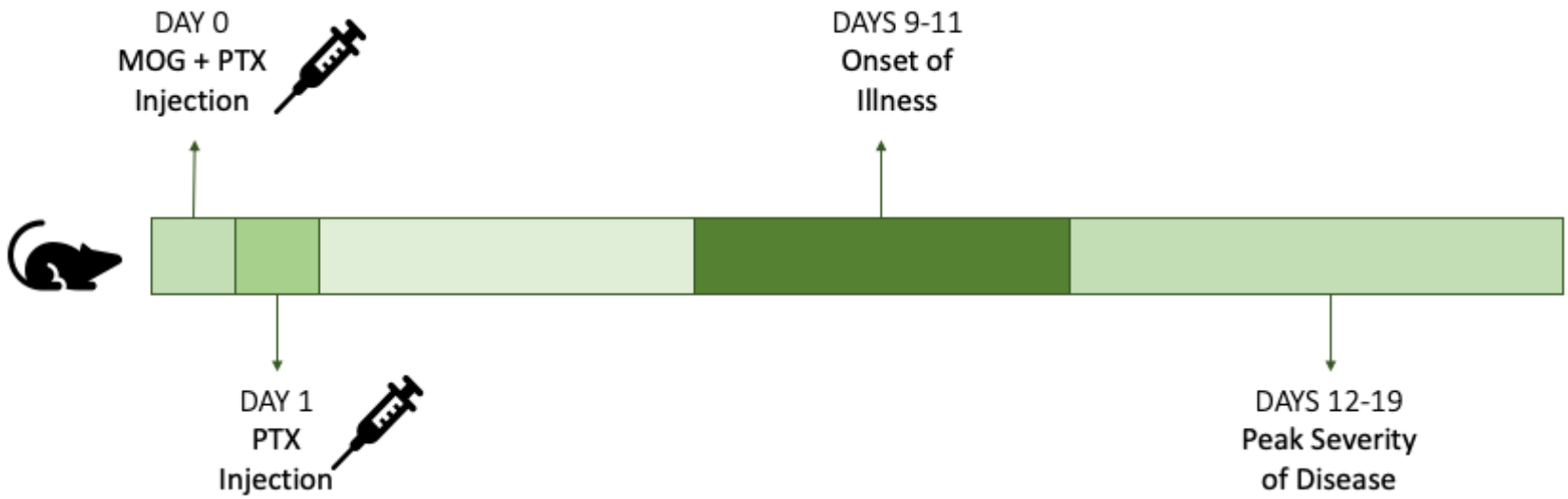

Figure 4. EAE induction timeline schematic. C57BL/6 mice injected intraperitoneally at two sites (upper back between front limbs and lower back between hind limbs) with 0.1 mL/site on day 0 with MOG_{35-55} in complete Freund's adjuvant (CFA). They also received a 0.1 mL pertussis toxin (PTX) solution intraperitoneal injection. On day 1, mice were injected again with only the PTX solution (0.1 mL). Onset of EAE disease occurs around day 9-11 followed by peak severity around day 12-19, depending on PTX dose.

2.1.3 EAE Clinical Scoring

Table 2.1 EAE Clinical Scoring Guidelines

Score	Clinical Observations
0.0	No obvious changes in motor function
0.5	Tip of the tail is limp
1.0	Limp tail – when picked up by base of tail, entire tail drapes over finger; no movement in tails
1.5	Limp tail and hind limb inhibition – when the mouse is dropped on a wire rack, one hind limb falls through consistently; walking is slightly wobbly
2.0	Limp tail and hind limb weakness – when picked up by base of tail, hind limbs are held close together; clear wobbly walk; dragging of toes in one foot
2.5	Limp tail and dragging of hind limbs – both hind limbs have some movement but both feet are dragging; no movement in one hind limb but movement in the other
3.0	Limp tail and complete hind limb paralysis – limbs are apart and both are dragged behind the body; one or both hind limbs are able to "paddle" but cannot move forward of the hind hip
3.5	Limp tail, complete hind limb paralysis and unable to right itself – limbs are together and dragged behind the body; when mouse is placed on its side, it is unable to right itself
4.0	Limp tail, complete hind limb paralysis and partial front limb paralysis – mouse moves minimally around the cage but appears alert and feeding
4.5	Limp tail, complete hind limb paralysis, partial front limb paralysis and no movement around cage – mouse is not alert or feeding
5.0	Moribund

If mice scored a 4.0 on two consecutive days or scored 4.5 or 5.0 on any given day, they were euthanized and received a score of 5.0 the rest of the experiment. Mice were assessed in a blinded manner daily (prior to treatment) for changes in clinical score. Mice were identified using markings on their tails.

2.2 Acute Intermittent Hypoxia (AIH)

Mice started daily treatment with AIH when they reached the mandatory pre-determined clinical score in each experimental group. Each mouse was randomly assigned to a treatment group (AIH or Normoxia) on a rolling basis as they reached this score. During treatment, mice were placed in custom-made plexiglass chambers (no more than 3 in a chamber) with paper towel on the floor. The AIH protocol is performed under automated control and delivers continuous gas flow to the chambers. For the AIH treatment group, the chambers received continuous flow of medical air (21% O_2) for 5 min alternating with hypoxic oxygen (11% O_2) for 5 min. Each switch between different O_2 levels was preceded by a 1 min purge in which the chambers expelled all air and re-filled with the correct O_2 levels for the next cycle condition. The complete daily treatment consisted of 10 cycles, for a total of 2 hrs of alternating 21% O_2 and 11% O_2.

Control EAE mice were in adjacent chambers at the same time under continuous normoxia. For this Normoxia treatment group, the chambers received continuous flow of medical air (21% O_2) for the total 10 cycles/2 hrs of treatment. O_2 and CO_2 levels within the chambers were repeatedly monitored using an oxygen/carbon dioxide analyzer (Quantek Instruments, Inc.; Grafton, MA, USA; model #902P) to ensure O_2 levels were at 11% or 21% and CO_2 levels remained at 0.01%-0.02%. Each treatment (AIH or Normoxia) mouse underwent a total of 7 days of treatment.

2.3 Experimental Design

A total of 80 C57BL/6 mice (5 mice never reached required clinical scores and did not go on to receive treatment or be assessed further) received treatment and were used in this project to determine the effects that AIH treatment might have upon clinical scores and pathological aspects of EAE. The mice were divided amongst 2 experimental groups to be assessed: Group 1 (treatment begun at first sign of disease onset; n = 20) and Group 2 (treatment begun at near peak EAE disease; n = 22). Naïve mouse tissue was also collected for immunohistochemistry (n = 26). An additional group of naïve mice (n = 12) received 4 days of AIH (n = 6) or Normoxia (n = 6) treatment and tissue was collected 18 hours after the last treatment for preliminary data.

GROUP 1: Treatment begun at first sign of disease onset (score = 1)

EAE was induced in 20 mice. At the first sign of disease onset (score = 1; limp tail), treatment was initiated (AIH: n = 10; Normoxia: n = 10). Treatment ran for 7 consecutive days. The mice were scored for an additional 7 days post-last treatment and sacrificed on the 7th day post-treatment. Tissue was collected for immunohistochemistry (n = 20).

GROUP 2: Treatment begun at near peak EAE disease (score = 2.5)

EAE was induced in 22 mice. When the mice reached a near peak EAE disease clinical score of 2.5 (limp tail and dragging of hind limbs), treatment was initiated (AIH: n = 11; Normoxia: n = 11). Treatment ran for 7 consecutive days. The mice were scored for an additional 7 days post-last treatment and sacrificed on the 7th day post-treatment. Tissue was collected for immunohistochemistry (AIH: n = 7; Normoxia: n = 8). 7 mice were used strictly for clinical score analysis (tissue was collected for western blots to be completed post-thesis defense).

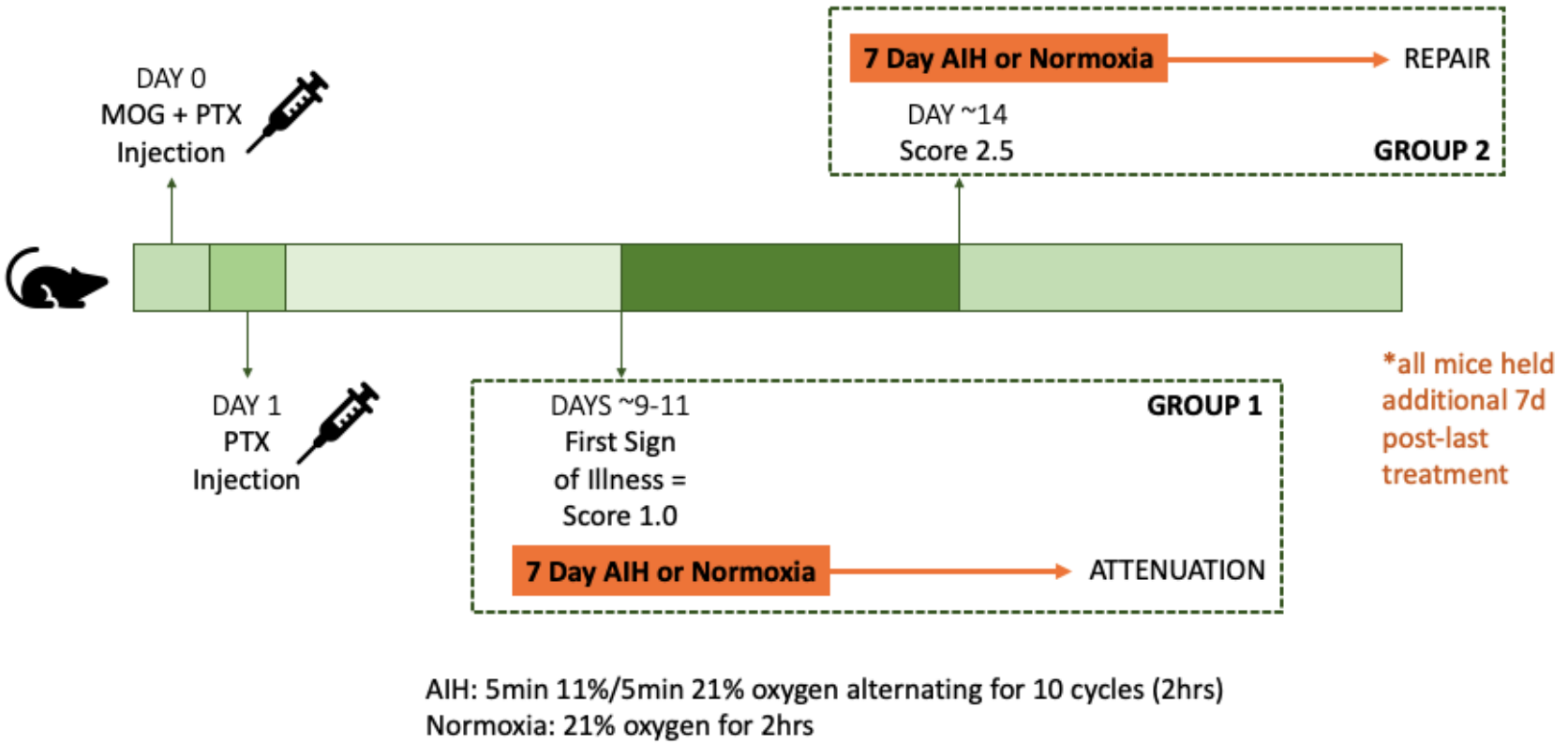

Figure 5. Experimental design timeline. C57BL/6 mice were induced with EAE with onset of disease occurring at about day 9-11 and peak around day 14. Group 1 began treatment at EAE onset of disease and received 7 days of either AIH or Normoxia treatment. Group 2 began treatment at near peak EAE disease and received 7 days of either AIH or Normoxia treatment to determine effects of AIH on prevention and attenuation of EAE disease. AIH treatment involved 10 cycles (2 hrs) of 11% oxygen for 5 min alternating with 21% oxygen for 5 min to assess effects of AIH on repair of EAE disease. Normoxia treatment animals received continuous 21% oxygen for 2 hrs. AIH and Normoxia treatment cycles ran at the same time. All mice were held an additional 7 days post-last treatment

2.4 Tissue Collection

Brains and spinal cords were harvested from animals on day 7 post-last treatment. For fixed-frozen tissue collection (n = 76), animals were euthanized with euthanyl forte (Bimeda-MTC Animal Inc; Cambridge, ON, Canada)/lidocaine (Pfizer Canada; Kirkland, QC, Canada) mix (72mg/kg euthanyl forte; 8mg/kg lidocaine) overdose and transcardially perfused with 20 mL of 0.1 M PBS followed immediately by 40 mL of 4% paraformaldehyde. Brains and spinal cords were removed and post-fixed in 4% paraformaldehyde for approximately 24 hrs at 4°C. They were then cryoprotected in 20% sucrose for either 24 hrs (spinal cords) or 48 hrs (brains) at 4°C. Following cryoprotection, tissue was embedded in optimal cutting temperature (OCT) compound (Fisher HealthCare; Houston, TX, USA; cat. #23-730-571), frozen in isopentane and stored at -80°C until processing. Spinal cords were embedded in both longitudinal (cut into cervical, lumbar and cauda equina) and transverse (cut to include cervical and lumbar regions) orientations in the same tissue block. For tissue collected for protein analysis (western blot; n = 7), animals were transcardially perfused with 0.1 M PBS. Tissue collected was placed in Tissue Protein Extraction Reagent (T-PER; Thermo Scientific; Rockford, IL, USA; cat. #78510) combined with cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche; Mannheim, Germany; cat. #4693159001) and immediately frozen and stored at -80°C until needed.

2.5 Histochemistry – Immunofluorescence (IF)

Fixed-frozen spinal cord tissue was cut at 10 μm on a Microm cryostat at -20°C. Care was taken to mount treatment (AIH or Normoxia) and control (Naïve) tissue on the same slide so as to allow for parallel processing and quantification of immunofluorescence (IF) signal as normalized to Naïve on that slide. Prior to processing for IF, slides were air-dried and acclimated to room temperature for 15 min. Slides were placed briefly onto a medium heat hot plate to ensure tissue adhered to the slide prior to the start of staining protocol. They were then washed 3 times x 10 min in 0.1 M PBS before being blocked with SeaBlock blocking buffer (Thermo Scientific; Rockford, IL, USA; cat. #37527) for 1 hr at room temperature in a humidified chamber. Blocking buffer was removed from slides and primary antibodies were applied to the slides and left to incubate overnight at 4°C in a humidified chamber. Primary antibodies (Table 2.2) were diluted in 0.1% Triton-X in 0.1 M PBS, 10% SeaBlock. The following day, slides were washed 3 times x 10 min in 0.1 M PBS and secondary antibodies were applied to the slides for a 1 hr incubation at

room temperature in a dark, humidified chamber. Secondary antibodies (Table 2.3) were diluted in 0.01% Triton-X in 0.1 M PBS, 1% SeaBlock. Slides were washed 3 times x 10 min in 0.1 M PBS in the dark and mounted with coverslips using Fluoroshield with nuclear DAPI stain (4',6-diamidino-2-phenylindole) mounting media (Abcam; Cambridge, UK; cat. #104139). Additional control slides were processed for IF without primary antibodies (diluent alone) for each IF marker used in this thesis. This was done to ensure that there was no nonspecific staining by the secondary antibodies used.

2.6 Data Analysis

2.6.1 EAE Clinical Score Analysis

Animals were scored daily (prior to receiving treatment on days once treatment began) based on clinical symptoms as per Table 2.1. Scores were averaged among treatment groups (AIH or Normoxia) per day. Statistical analysis was performed using *t*-tests (GraphPad Prism 5) to determine differences between treatment groups on each day. Values in figures are expressed as mean +/- standard error of the mean (SEM). Results achieved statistical significance at a *p*-value < 0.05.

2.6.2 Histochemical Analysis

As mentioned above, spinal cords from both treatment (AIH and Normoxia) groups and Naïve controls were mounted onto the same slide to ensure processing under identical conditions and to avoid slide to slide variabilities in IF when quantifying. Tissue sections were visualized, and pictures were acquired under identical exposure conditions using a Zeiss Axio Imager M.1 fluorescence microscope (20X and 40X objectives) and Northern Eclipse v7.0 software. Demyelinated areas were identified using the presence of DAPI and/or ED-1 positive staining (Figure 6). Data collection was carried out using FIJI ImageJ software[163]. Statistical analysis was performed using either a one-way ANOVA with Bonferroni's post hoc test or *t*-tests (GraphPad Prism 5) to determine differences between means. All values in figures are expressed as mean +/- standard error of the mean (SEM). Results achieved statistical significance at a *p*-value < 0.05.

Alterations in expression levels for markers of repair and axon protection were quantified in the following manners: (i) for quantification of MBP and PDGFRα, IF levels were measured

within highly inflamed areas/regions of interest (ROI) identified by either DAPI and/or ED-1; (ii) to quantify Caspr+ve paranodal regions within presumed nodes of Ranvier that were formed in the highly inflamed ROI or normal white matter (Naïve controls), punctate paired Caspr+ve paranodes were counted in the pre-determined ROI; (iii) all macrophage/microglia markers (excluding ED-1) were quantified using masks that identified ED-1+ve macrophages/microglia (Figure 7) on tissue sections dually processed for ED-1 and the macrophage/microglial marker of interest. First, a ROI was determined using DAPI and a mask that identified ED-1 positive cells was created within that ROI (ROI is white). The mask was created by thresholding the ED-1 labelled image so that activated macrophages/microglia were white, and the background was black. Any IF outside the ROI DAPI/ED-1 mask is then blacked out to match the background so that only IF within the ED-1 mask was quantified for the marker of interest. The ED-1 mask was then laid over the image taken of the marker of interest from the dually processed IF section and the IF signal for the second marker (iNOS, TNFα, CD206 or activin-A) within the defined ED-1 mask was measured; (iv) to quantify ED-1, a ROI was first created that included the white matter tract area surrounding the ED-1+ve inflamed area, ED-1 was then thresholded (ED-1 stained white) and fluorescence outside ROI was blacked out. A percentage of the area that was ED-1+ve within that ROI was calculated. All area measurements from the same animal were added together and divided by the total ventral lumbar spinal cord white matter area to determine the percent area occupied by ED-1 in the white matter tracts; (v) lastly, for the quantification of the axon protective marker, SMI31, that detects phosphorylated neurofilaments (pNF), a similar protocol to the macrophage quantification was followed. A ROI using DAPI to identify inflamed areas was first created. Both SMI31 and βIII tubulin images were thresholded (stained areas are white) and fluorescence outside ROI was blacked out. On the βIII tubulin image, βIII+ve areas were selected and used to create a mask that was then laid over the corresponding SMI31 image. The percentage of the axonal area defined by the βIII mask that had pNF (SMI31 +ve IF) was then determined. All macrophage/microglia markers were normalized to Normoxia control as there is virtually no ED-1 positive cells in Naïve spinal cord tissue. All other IF markers were normalized to Naïve control.

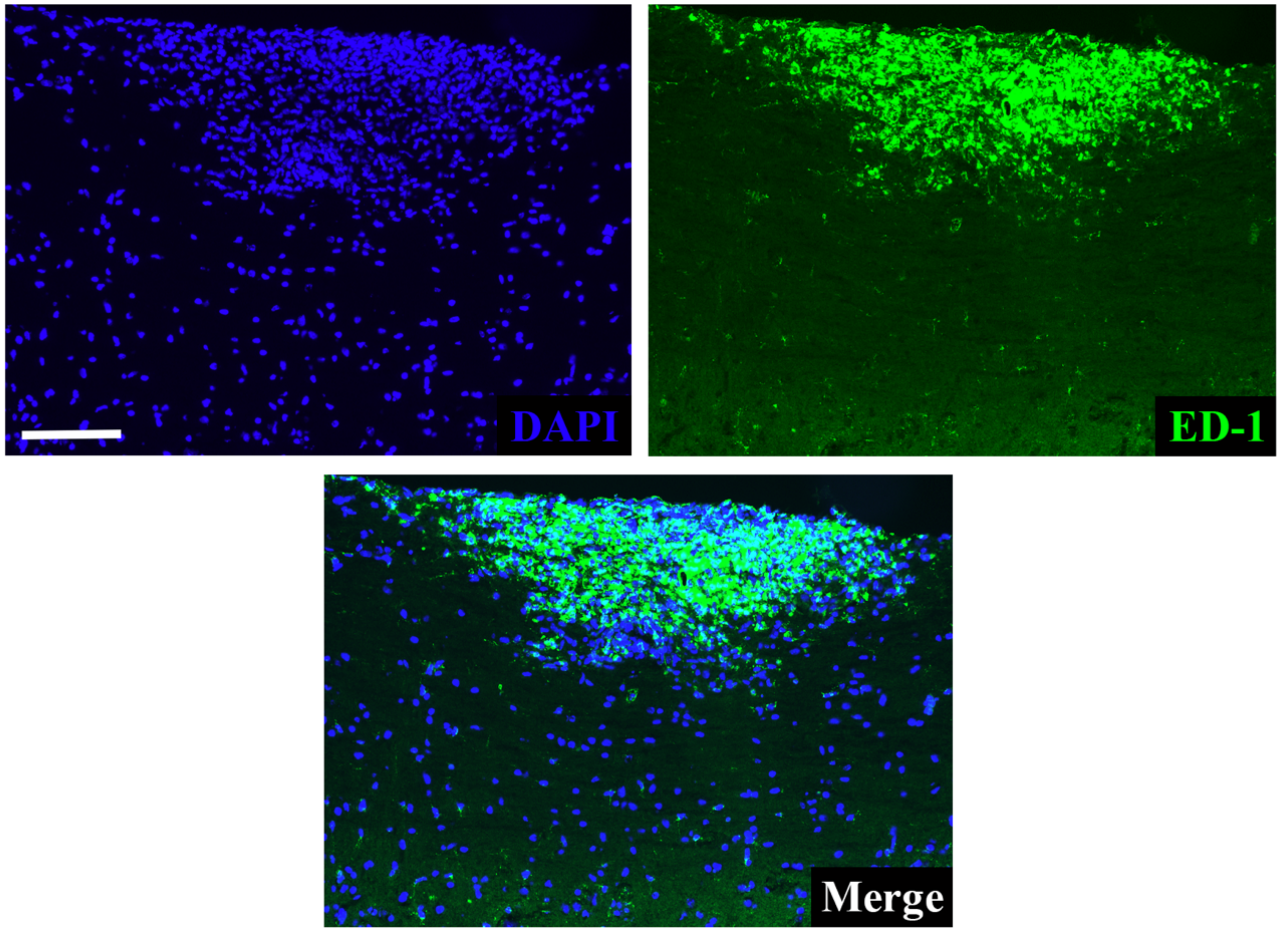

Figure 6. Regions of interest (ROI) in EAE animals detected by elevated inflammation colocalize with high density DAPI+ve areas. Representative longitudinal sections of spinal cord from lumbar enlargement (from EAE mice) processed for markers as indicated to detect nuclear DAPI and activated macrophages/microglia (ED-1). High density DAPI+ve regions coincide with ED-1+ve areas and thus, can also be used to delineate ROI.

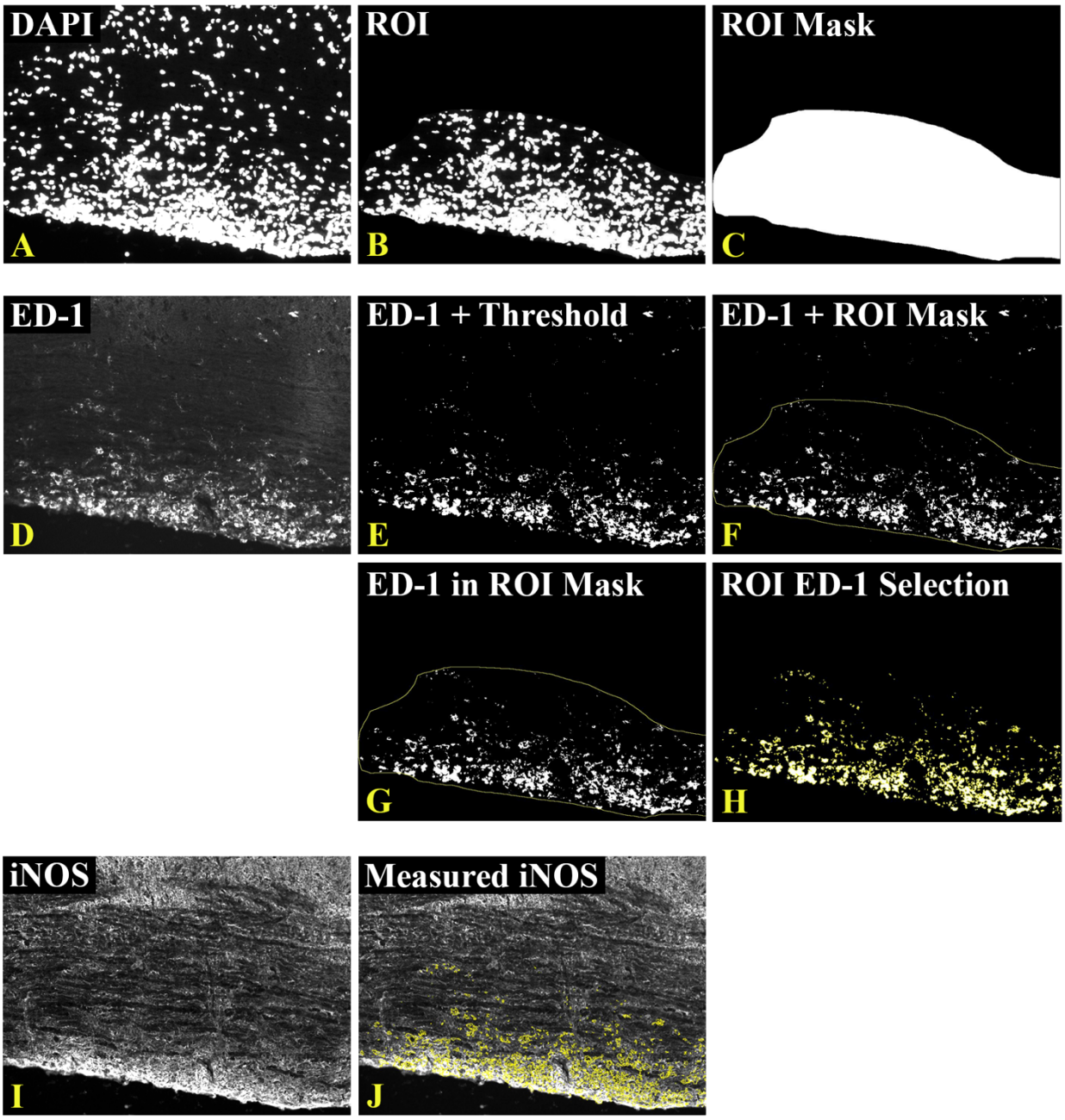

Figure 7. Example immunofluorescence quantification with use of mask. Representative longitudinal sections of spinal cord from lumbar enlargement (from EAE mice) dually processed for immunofluorescence (IF) to detect nuclear activated macrophages/microglia (ED-1) and pro-inflammatory iNOS protein and stained with the nuclear marker DAPI. **A.** DAPI labelled image. **B.** Region of interest (ROI) selected based on high density DAPI+ve area (high density ED-1+ve regions coincide with DAPI-1+ve areas and thus can also be used to delineate ROI; in cases where slides with dually processed with ED-1, ED-1 was used to indicate ROI). Area outside of

ROI is blacked out. **C.** Mask created using DAPI+ve ROI (white area = ROI). **D.** ED-1 labelled image. **E.** Threshold applied to ED-1 image in which white areas colocalize with activated macrophages/microglia IF signal as detected by ED-1. **F.** ROI mask selection placed over threshold ED-1 image so that only marker of interest is only quantified within ED-1+ve immune cells in the ROI. **G.** ED-1+ve immune cells outside of ROI are painted black so that they are not included in quantification. **H.** ED-1+ve ROI is selected. **I.** iNOS labelled image. **J.** ED-1+ve ROI selection placed over iNOS image to determine iNOS IF within activated macrophages/microglia mask (ED-1+ve iNOS levels).

2.7 Antibodies

2.7.1 Primary Antibodies

Table 2.2 Primary Antibodies Used for Immunofluorescence (IF).

Target	Species	Catalog Number	Dilution
Activin-A	Goat	R&D Systems #AF338	1:25
BDNF	Chicken	Promega #G1641	1:75
β-Tubulin III	Rabbit	Sigma-Aldrich #T2200	1:500
Caspr	Rabbit	Abcam #ab34151	1:4500
CD206	Goat	Santa Cruz #SC-34577	1:100
CD68	Rat	Abcam #ab53444	1:500
CD68	Rabbit	Abcam #ab125212	1:600
GAP43	Mouse	Ascites #9-1E12	1:750
HIF1α	Rabbit	GeneTex #GTX127309	1:200
iNOS	Rabbit	Sigma-Aldrich #N7782	1:500
MBP	Rat	NovusBio #NB600-717	1:20
PDGFRα	Goat	R&D Systems #AF1062	1:75
SMI31	Mouse	Cedarlane #SMI-31R	1:1000
TNFα	Rabbit	Abcam #ab6671	1:75

*Note: CD68 and ED-1 are used interchangeably in this thesis.

2.7.2 Secondary Antibodies

Table 2.3 Secondary Antibodies Used for Immunofluorescence (IF).

Target	Species	Catalog Number	Dilution
Goat Alexa488	Mouse	Abcam #150117	1:1000
Mouse Alexa488	Rat	Jackson ImmunoResearch #212-545-106	1:1000
Donkey CY3	Chicken	Jackson ImmunoResearch #703-165-155	1:1000
Donkey CY3	Rabbit	Jackson ImmunoResearch #711-166-152	1:3000
Donkey DyLight488	Goat	Jackson ImmunoResearch #705-485-003	1:1000

CHAPTER 3: RESULTS

3.1 Impact of AIH on EAE Clinical Disease Scores

AIH has been shown to have a remarkable impact on nervous system plasticity and functional outcomes in other nervous system pathologies that also require repair, such as spinal cord injury and peripheral nerve injury[153–155,160,164]. Because of this, I sought to determine whether it might serve to either attenuate disease progression or enhance repair processes in the EAE mouse model of MS. To examine this, the impact of one-week daily AIH treatment or Normoxia control treatment on the clinical disease severity scores was assessed in two groups of female EAE mice. Group 1 began treatment at onset of disease to determine if AIH could attenuate EAE disease development and progression; while Group 2 began treatment near peak of disease to determine if AIH would be able to slow or reverse EAE disease progression and/or effect better repair. Clinical scores were recorded for each animal until the day of tissue collection which occurred 7 days after the last AIH or Normoxia treatment.

3.1.1 AIH begun at first sign of disease does not mitigate disease progression

Group 1 mice began AIH (10 cycles of 5 minutes each alternating normal air [21% O_2] and 11% O_2) or Normoxia (10 cycles of normal air [21% O_2]) treatment when they reached a score of 1 (limp tail; about day 7-9 post injection), known as onset of disease. Treatment was administered once daily for a total of 7 days. Mice were scored for an additional 7 days post last treatment. The Normoxia group showed typical EAE disease progression with peak disease 4 days post onset of disease, partial recovery at 10 days post onset and reappearance of more severe clinical symptoms following recovery (Figure 8). The AIH group also showed peak of disease 4 days post disease onset and generally showed worse clinical scores than the Normoxia group post onset but none of these differed significantly (Figure 8). It appears that starting AIH treatment at onset of disease does not improve clinical outcomes in EAE mice.

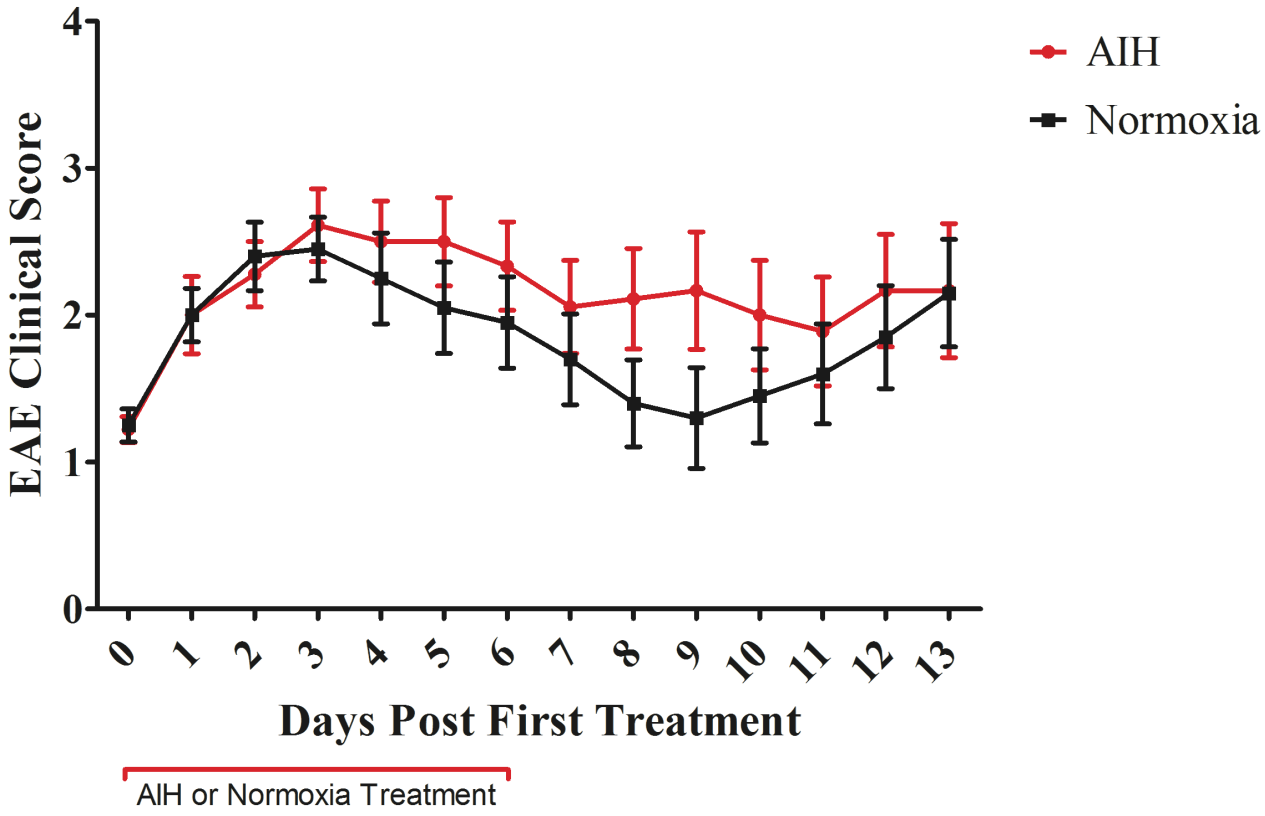

Figure 8. 7d AIH treatment started at EAE onset of disease score of 1.0 did not significantly impact clinical score outcomes. Graph depicting no significant differences in EAE clinical scores between animals receiving daily Normoxia treatment and AIH treatment begun at onset of disease score = 1.0 for 7 days (day 0 – day 6). Mice were randomly assigned to treatment groups. N=9-10 EAE mice/treatment group; mean +/- s.e.m.; *t*-test (significance at $p<0.05$).

3.1.2 AIH begun at near peak disease significantly improves disease scores

EAE mice in Group 2 began AIH or Normoxia treatment when they reached a score of 2.5 (limp tail and dragging of hind limbs; about day 12 post-injection), known as near peak disease which would be the equivalent of starting treatment after one had had an MS attack with noticeable deficits. Treatment was administered once daily for a total of 7 days. Mice were scored for an additional 7 days post-last treatment. The Normoxia group showed typical EAE disease progression with near peak of disease 2 days post initiation of treatment, minor recovery at 7 days post initiation of treatment and very slight worsening of symptoms following recovery (Figure 9). In contrast, the AIH group presented with peak of disease 2 days post disease onset when AIH treatment was started and scored significantly better than their Normoxia counterparts evident as early as 2 days with a continual improvement evident as long as 10-11 days post-initiation of AIH treatment. This improvement back to near normal clinical scores was followed by a slight worsening of symptoms in the last 4 days of scoring, possibly due to the fact that AIH treatment was only delivered for 7 days. However, there was no statistical significance within the AIH treatment group between day 9 and day 13, which saw the greatest increase in clinical scores after the last treatment. This indicates that while there was a trend toward worse clinical scores, it was not significant. Thus, it appears that Normoxia treated EAE mice developed significantly worse EAE disease than their AIH treated counterparts (Figure 9). Furthermore, the return of clinical scores to a near normal state in response to AIH suggested that it might be inducing an enhanced repair state.

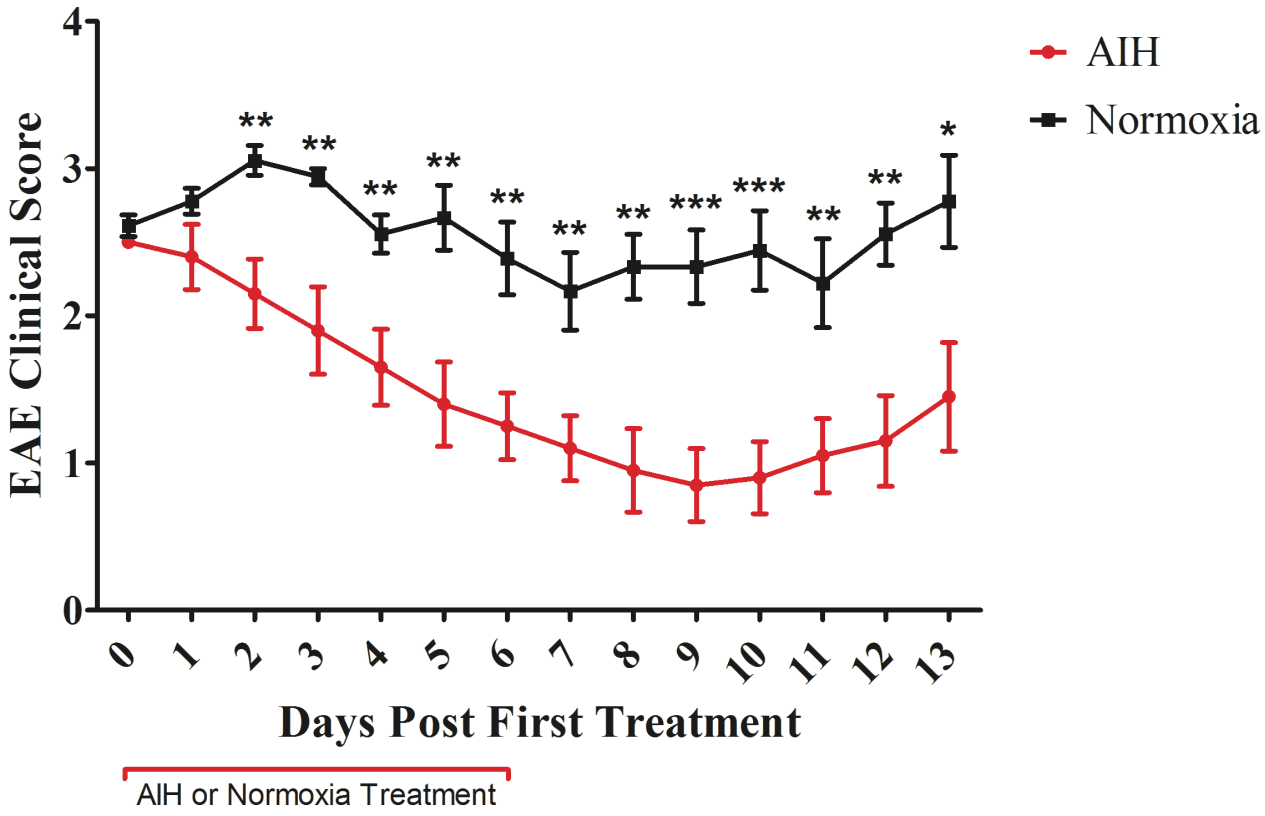

Figure 9. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a remarkable impact on clinical score outcomes. Graph depicting significant differences in EAE clinical scores for animals receiving daily Normoxia treatment and AIH treatment begun at near peak score = 2.5 for 7 days (day 0 to day 6). Mice randomly assigned to treatment group. N=9-10 EAE mice/treatment group; mean +/- s.e.m.; *t*-test (significance at *p<0.05; **p<0.01; ***p<0.001).

*Note: Due to the time constraints of this MSc thesis and the remarkable impacts of AIH observed in Group 2, it was decided that the histopathological investigations would focus strictly on Group 2, in which AIH treatment was started at near peak of disease (score = 2.5) and had dramatic and significant effects on function. While this thesis did not delve further into Group 1 outcomes in which treatment was started at onset of disease (score = 1), future research in our lab will elucidate the histopathological correlates of Group 1 animals, as it is important to gain insight as to why this timing of intervention did not mitigate the disease state and whether this was associated with specific cellular states.

3.2 Impact of AIH on Expression of Plasticity-Associated Genes in Naïve Mice

Molecules associated with plasticity are important in the activation and maintenance of remyelination and repair. There is evidence that many of these proteins, including BDNF, GAP43 and HIF1α, are activity-dependent and are often up-regulated in response to increased neural activity[165]. In particular, neural activity enhancing treatments such as exercise or ES have been observed to enhance plasticity-associated genes[117,166]. In previous research, AIH treatment has been observed to increase LTF in phrenic motor neurons (i.e. increased neural firing in response to AIH therapy) and to also enhance levels of plasticity-associated proteins but these observations were made in regard to spinal cord injury[153,158]. Thus, before beginning my thesis work, I performed preliminary experiments to examine if there were similar increases in plasticity markers in Naïve C57BL/6 control mouse tissue using the AIH parameters being employed in this study.

It should be noted that for all plasticity associated IF markers examined, qualitative observations, that were made by at least two observers, were reproducible between all animals in each experimental group. All images were captured under identical conditions so as to accurately depict relative changes in fluorescence signal between experimental groups.

3.2.1 AIH treatment enhances plasticity-associated gene expression

Naïve C57BL/6 mice were administered 4 days of either Normoxia (n = 6) or AIH treatment (n = 6). All mice were perfused 18 hours following the last treatment to allow sufficient time for any mRNAs induced by the treatment to be translated into protein and examined using IF histochemistry. In the Normoxia control tissue, neuronal and non-neuronal BDNF, GAP43, and HIF1α were all qualitatively observed at low levels in the ventral region of the lumbar spinal cord (Figure 10). Qualitative analysis of all slides revealed that 4 days of AIH treatment resulted in increased levels of neuronal BDNF, GAP43 and HIF1α protein expression/IF signal in the Naïve C57BL/6 mouse lumbar ventral spinal cord tissue. Elevated levels of GAP43 and HIF1α were also detected in the neuropil surrounding the motor neurons, with changes in GAP43 more evident in this area than in the neurons themselves (Figure 10). Thus, C57BL/6 mice respond in a similar manner to AIH treatment as do the Lewis rats used in previous spinal cord injury studies[149,153,154]. AIH would therefore be predicted to impact the expression of plasticity associated genes in EAE mice used in this study.

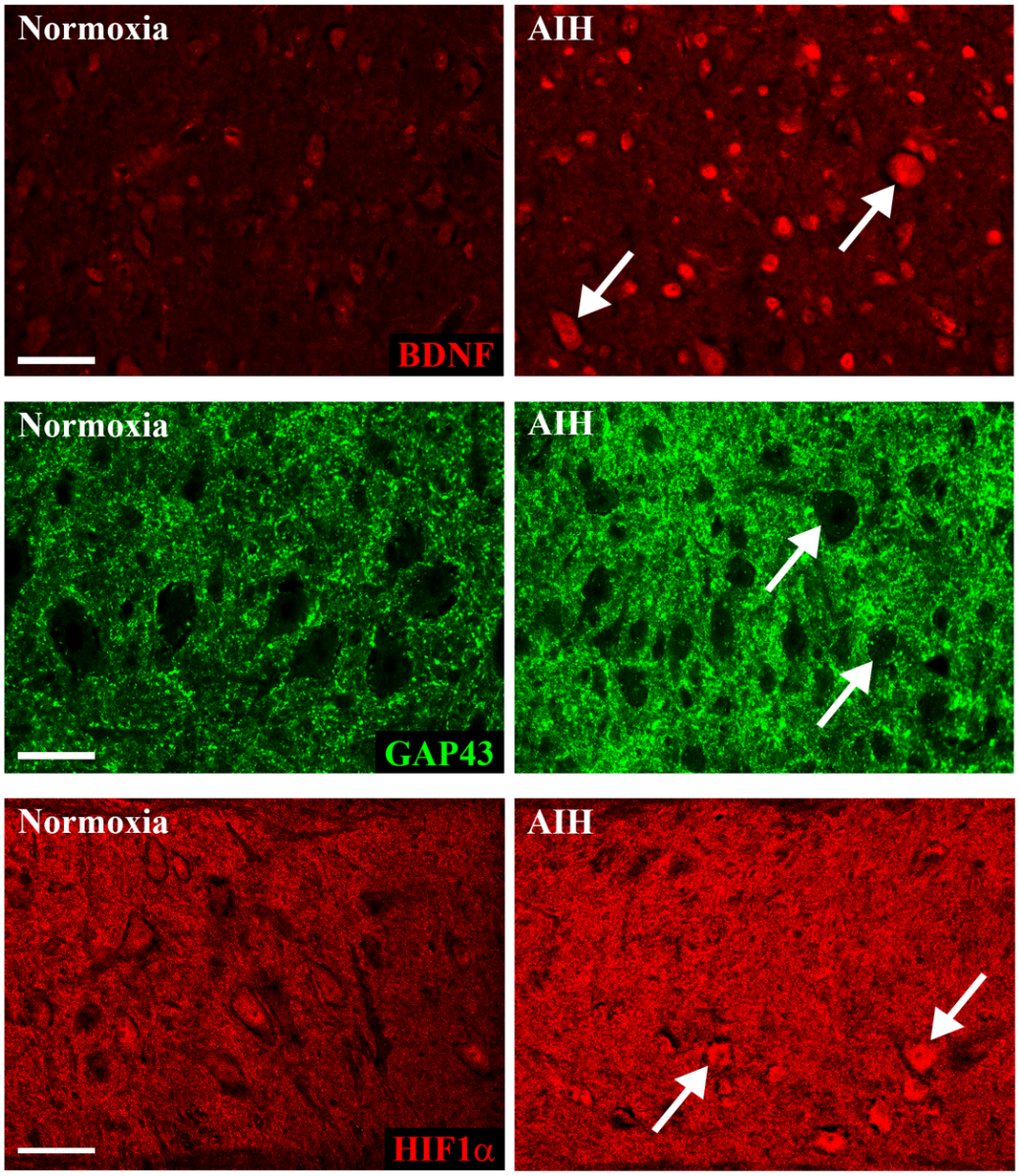

Figure 10. Preliminary data – 4d of daily AIH versus Normoxia treatment in Naïve C57BL/6 mice followed by perfusion 18 hours later results in elevated BDNF, GAP43 and HIF1α protein expression. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on the same slide and processed for immunofluorescence to detect brain-derived neurotrophic factor (BDNF; top), growth-associated protein 43 (GAP43; middle) and hypoxia-inducible factor 1-alpha (HIF1α; bottom) with representative neurons identified with arrows. Note: 4d AIH treatment results in elevated levels of BDNF, GAP43 and HIF1α compared to Normoxia controls with elevated levels also detected in the neuropil for GAP43 and HIF1α. Scale bar = 100 μm.

3.3 Impact of AIH on Intrinsic Repair Processes

To examine the impact that one-week daily AIH treatment has on histopathological processes associated with intrinsic repair mechanisms, I used IF histochemistry to investigate multiple markers of intrinsic repair processes. Thus, one week of daily AIH treatment was compared to Normoxia treatment, both begun at near peak of disease (score = 2.5), with respect to enhancement of repair in EAE mice relative to Naïve control mice. It should be noted that for all intrinsic repair IF markers examined, qualitative assessments of relative changes in expression between treatment groups were reproducible for all animals in each experimental group. All images of ventral spinal cord from the lumbar enlargement were captured under identical conditions and blinded prior to quantification to allow for unbiased analysis.

3.3.1 AIH promotes an axon protective phenotype through the phosphorylation of neurofilaments

Axonal degeneration initiated either in the neuron or as a consequence of demyelination is a major contributor to functional deficits in MS[167]. Thus, approaches that put axons in a protected state when they are demyelinated is a strategy to mitigate axon loss until remyelination can occur. Alongside myelination, there is evidence that phosphorylation of neurofilaments is also an important factor in protecting axons. It is believed that phosphorylation of the neurofilaments protects the axon by increasing axonal caliber, which is a factor in determining myelination, and by making it more resistant to calpain-associated degradation[64,65,168]. Further, neurofilament phosphorylation is also modulated by myelination[61]. For example, in MS, there is significant neurofilament dephosphorylation following demyelination[100]. As such, the appearance of higher levels of pNF in areas of inflammation and demyelination would suggest that the axons are in a more protected state from degenerative events. Previously published work from our lab showed that increasing neural activity in nerves that had been focally demyelinated through ES resulted in a rapid and significant increase in pNF in the zones of demyelination[117]. Thus, the impact of AIH (another strategy to increase neural firing) on levels of pNF in regions of inflammation in the white matter tracks of the lumbar spinal cord of EAE mice +/- AIH was examined.

In longitudinal sections of Naïve controls, robust linear pNF expression can be observed within presumed axons, as detected by the pNF antibody, SMI31. This signal was indeed within axons as it colocalized with another axonal cytoskeletal marker, βIII tubulin whose expression

has not been reported to change in EAE, unless the axon degenerates[169]. Thus, βIII tubulin IF can be used to indicate an axon's presence even if it no longer expresses pNF.

ROI in the lumbar white matter tracts with high levels of inflammation were identified by DAPI intense regions that had been shown to have coincident high levels of ED-1+ve activated macrophages/microglia (Figure 6). Changes in pNF were quantified within axons in the ROI by dual processing for βIII tubulin IF to identify the axons. Then a mask was created that selected βIII tubulin positive structures within the inflamed ROI and overlaid on the corresponding SMI31/pNF to quantify the amount of SMI31+ve IF signal in those axons.

Within the inflamed white matter ROI in the Normoxia group, axons are still detected although they appear less organized, likely due to the high degree of inflammation (Figure 11). There was a dramatic reduction in pNF expression compared to the AIH treatment group within inflamed white matter EAE tissue 7 days following the last Normoxia treatment started at EAE near peak of disease, score = 2.5. The levels of pNF at this point were only 53.5% that of Naïve (15.9337 +/- 0.9735 vs. 29.7798 +/- 1.477; Figure 11). This reduction in pNF expression correlates dephosphorylation of neurofilaments with EAE-associated inflammation observed on the adjacent section processed for ED-1 IF and regions of demyelination observed on subsequent slides (data not shown).

In contrast, EAE animals receiving 7 days of AIH treatment did not show the same degree of reduced pNF expression, nor did the they have the same degree of inflammation as detected on the adjacent slide processed for ED-1 or demyelination (data not shown). Further, the axons within the AIH ROI were better organized than those observed in the Normoxia ROI. The AIH treatment group had significantly higher levels of SMI31 IF within the βIII tubulin +ve axons in the ROI, with levels returning to 81.3% that of Naïve control tissue SMI31 levels (24.2093 +/- 1.577 vs. 29.7798 +/- 1.477; Figure 11).

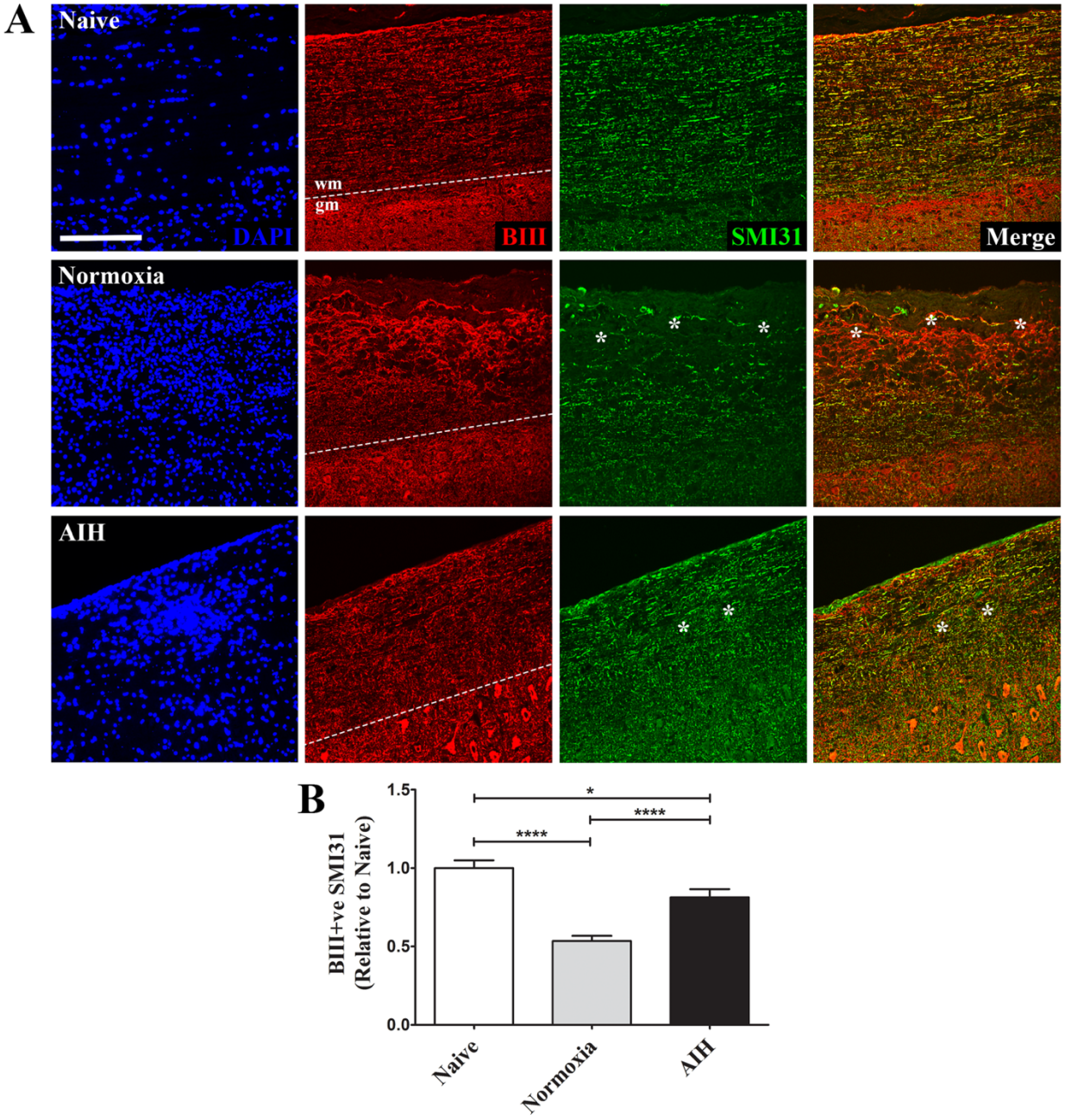

Figure 11. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a significant impact on promotion of axon protective phenotype. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect the axon protective phenotypic marker phosphorylated neurofilaments (pNF) using SMI31 antibody and axons using βIII tubulin antibody as indicated. DAPI intensive areas in experimental tissue delineate regions of interest (ROI) where SMI31 levels were quantified. Note: while the axons are still present (βIII tubulin),

Normoxia treated animals have reduced levels of pNF IF (white asterisks) while inflamed areas in the AIH group show more abundant levels of linear axons positive for pNF. **B.** Quantification of SMI31 levels as normalized to levels in Naïve controls confirms qualitative observations. N=7 mice/treatment with 28-46 ROI sampled per condition; mean +/- s.e.m.; ANOVA (*$p<0.05$; ****$p<0.0001$). Scale bar = 200 μm.

3.3.2 AIH promotes paranodal node of Ranvier appearance in manner consistent with nodal reorganization

When axons are demyelinated, the axonal proteins that normally cluster in the nodal, juxtaparanodal and paranodal regions of the Nodes of Ranvier disperse and only re-cluster in a punctate manner when the axons are remyelinated. In particular contactin-associated protein (Caspr) is complexed with contactin and targeted to the paranodes of axons when nodes of Ranvier reform and remyelination occurs, re-establishing a distinct staining pattern on either side of the node of Ranvier[170]. As a result, I decided to examine the degree of this punctate Caspr+ve paranodal IF staining bordering each side of the nodes of Ranvier to assess if AIH treatment may affect a greater degree of paranodal re-organization (versus Normoxia).

In Naïve mice, defined areas of the white matter tracts normally affected in EAE mice were selected and the area measured. I then counted the number of Caspr+ve paranodes that were visible in the defined area in order to determine the density of paranodes. In order to be counted, the Caspr IF had to be an intense punctate pairing with a small gap in between where the presumptive node of Ranvier would be located.

To assess the impact of one-week Normoxia or AIH treatment on the density of Caspr+ve paranodes in inflamed ROI, the ROI was first identified, and the area measured, followed by a counting of all Caspr+ve paranodes in the ROI in order to calculate a paranode density. EAE mice that received 7 days of Normoxia treatment at near peak EAE disease showed a significant decrease in paranode density with the appearance of the staining shifting from the punctate staining readily observed in the Naïve white matter tracts to a more diffuse distribution (Figure 12). In contrast, AIH treatment appeared to promote reorganization of the paranodes in areas of inflammation/ROI as evident by the increased density of Caspr+ve paranodes (Figure 12). The ratio shift in the approximate density of paired Caspr+ve paranodes from Naïve to Normoxia to AIH was 5.1 : 1.1 : 3.5, as reflected in the data normalized to Naïve in Figure 12. Increased

density of Caspr+ve paranodes in inflamed regions indicate that AIH treatment may have a beneficial effect on the re-establishment of node of Ranvier properties consistent with remyelination.

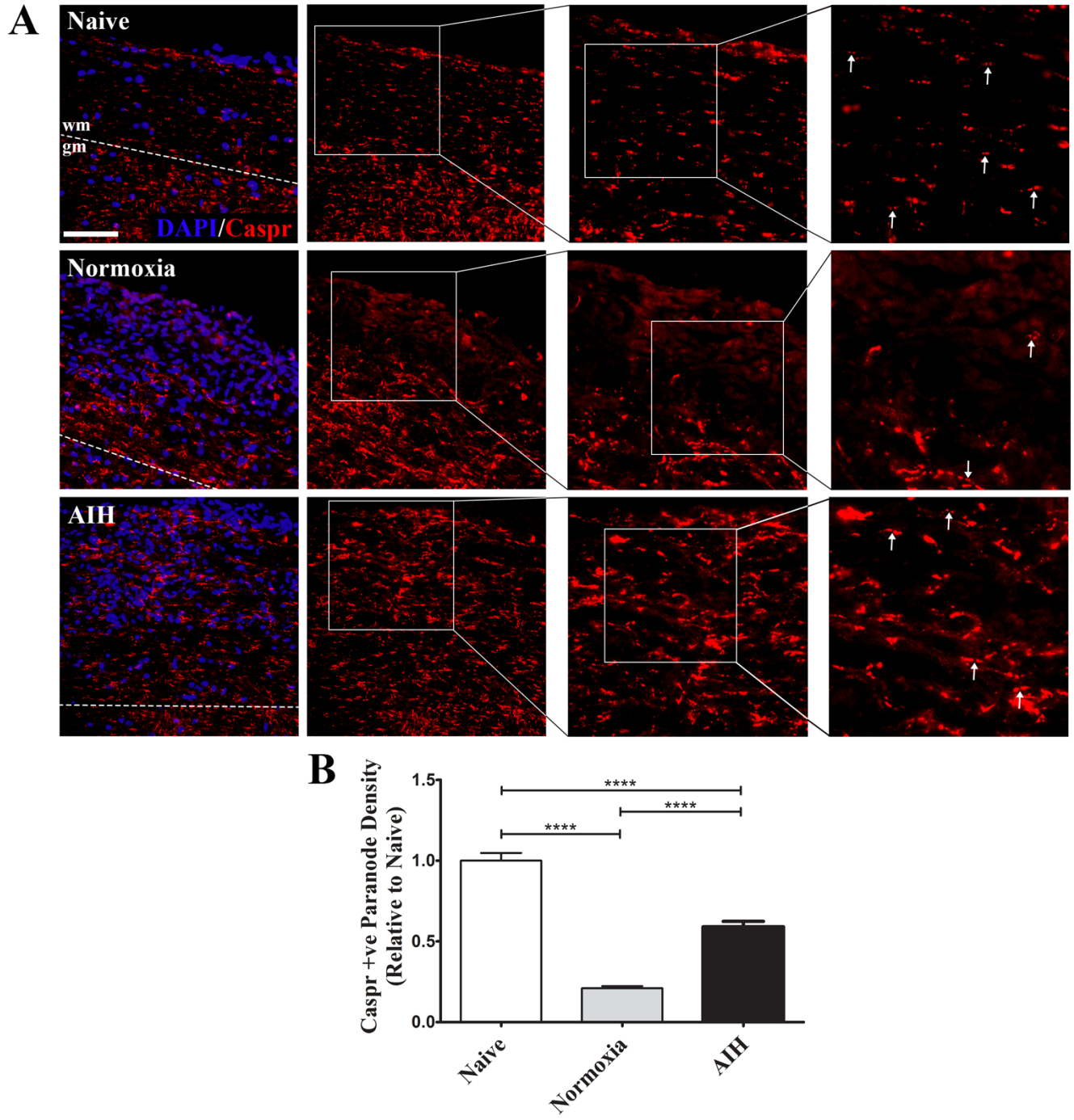

Figure 12. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a remarkable impact on the appearance and organization of paranodal regions of the nodes of Ranvier. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect punctate Caspr+ve paranodal regions in regions of inflammation (DAPI intense areas). AIH treatment promotes a significant increase in the numbers of Caspr+ve paranodes detected as

punctate densities on either side of presumed nodes of Ranvier (examples shown with arrows) detected in sites of inflammation/ROI. **B.** Quantification of Caspr+ve paranodal regions in ROI confirms qualitative observations. N=7 mice/treatment with 28-51 ROI sampled per condition; mean +/- s.e.m.; ANOVA (****$p<0.0001$). Scale bar = 200 μm.

3.3.3 AIH treatment results in increased MBP expression and reduced inflammation

To assess whether AIH treatment resulted in greater amounts of myelin/MBP being detected in inflamed ROI (regions that were likely demyelinated, initially) and whether this is associated with inflammation being more resolved, the sections analyzed were dually processed for ED-1 IF and DAPI to identify ROI and MBP IF as a reflection of overall myelin levels.

A consistent observation when examining tissue sections from similar levels of all experimental groups mounted on the same slide is that the AIH treated EAE mice tended to have much less inflammation (less ED-1+ve cells), as compared to Normoxia (Figures 13, 14). In the Normoxia group, high levels of inflammation were evident in both white and gray matter, while in AIH-treated mice, the limited inflammation that was observed appeared localized only to the white matter, suggesting that AIH mice had undergone significant resolution of the inflammation (Figure 13).

Longitudinal and transverse sections of lumbar spinal cord showed comparable qualitative results. The Naïve control spinal cord tissue showed high levels of immunostaining in the white matter for MBP with a predominantly linear pattern in the longitudinal sections. In the Normoxia group, there was an evident loss of MBP and its linear pattern indicating demyelination in the highly ED-1+ve ROI. Within these ROI even when high MBP IF was detected, the MBP tended to be highly punctate and colocalized with the ED-1 IF signal, indicating that it was likely myelin debris that had been taken up by phagocytic macrophages/microglia. Outside of this punctate MBP signal, there was a significant overall decrease in MBP levels, both qualitatively and quantitatively, in the Normoxia treatment group, consistent with extensive demyelination (Figures 13, 14). To ensure that this MBP+ve debris detected in ED-1+ve cells did not artificially bias the quantification of how much actual MBP associated with axons was found in regions of inflammation, a mask of the ED-1 positive cells in the ROI was created and overlaid on the corresponding MBP image to calculate how much of the MBP signal was actually within macrophages/microglia and this amount was subtracted from the total MBP levels in the ROI.

Thus, the remaining value should represent intact MBP in the region and not that associated with myelin debris.

Notably, 7 days of daily AIH treatment resulted in significantly higher levels of MBP IF as compared to the Normoxia group suggestive of greater myelination within regions of inflammation relative to Normoxia (Figures, 13, 14). The AIH-associated MBP IF signal was largely organized into linear patterns of MBP within the ROI, supporting that they had likely effectively remyelinated (Figure 14).

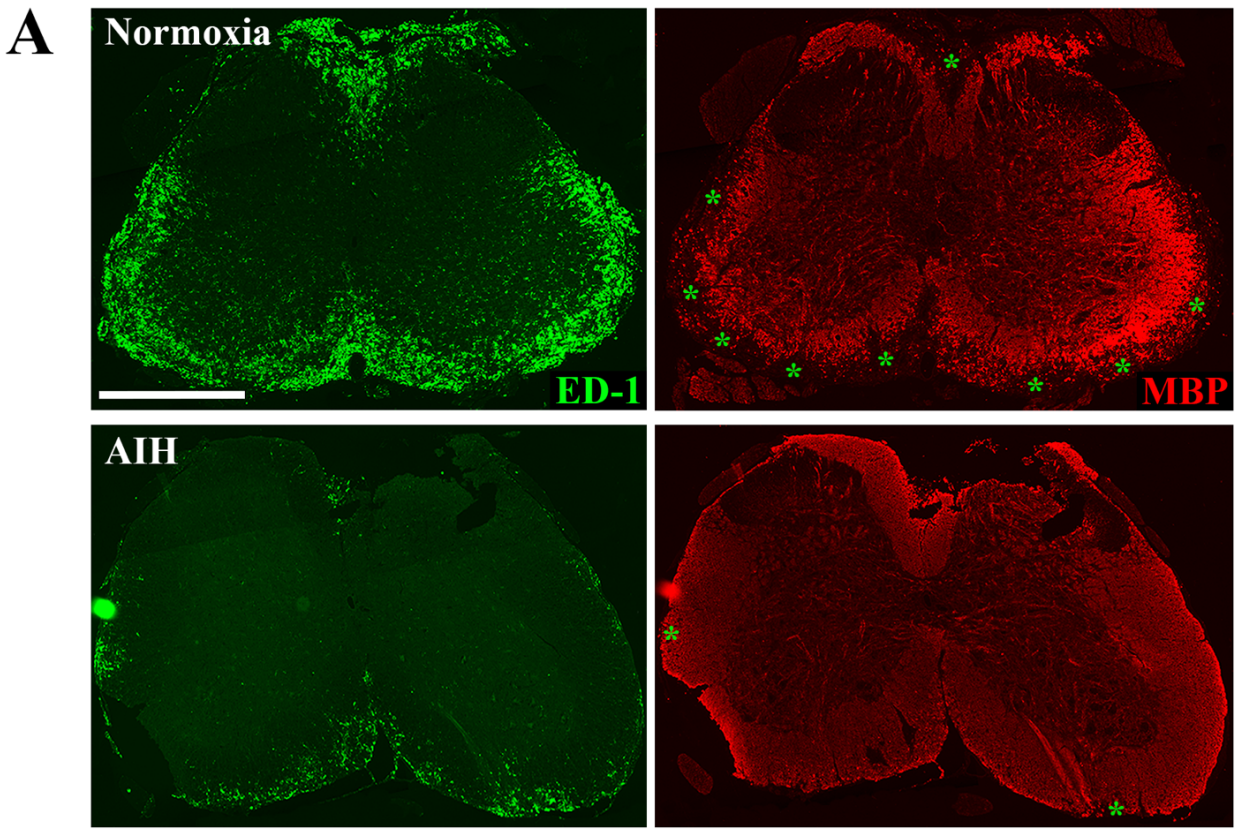

Figure 13. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a remarkable impact on pathological outcomes. Representative transverse sections of spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and myelin basic protein (MBP) in EAE mice. Note: 7d AIH treatment has a dramatic impact on the degree of inflammation (ED-1) and myelination (MBP; green asterisks) observed 14 days post-treatment initiation relative to Normoxia treated EAE animals. Relative levels of inflammation in white and gray matter regions are greatly reduced with AIH treatment. Scale bar = 500 μm.

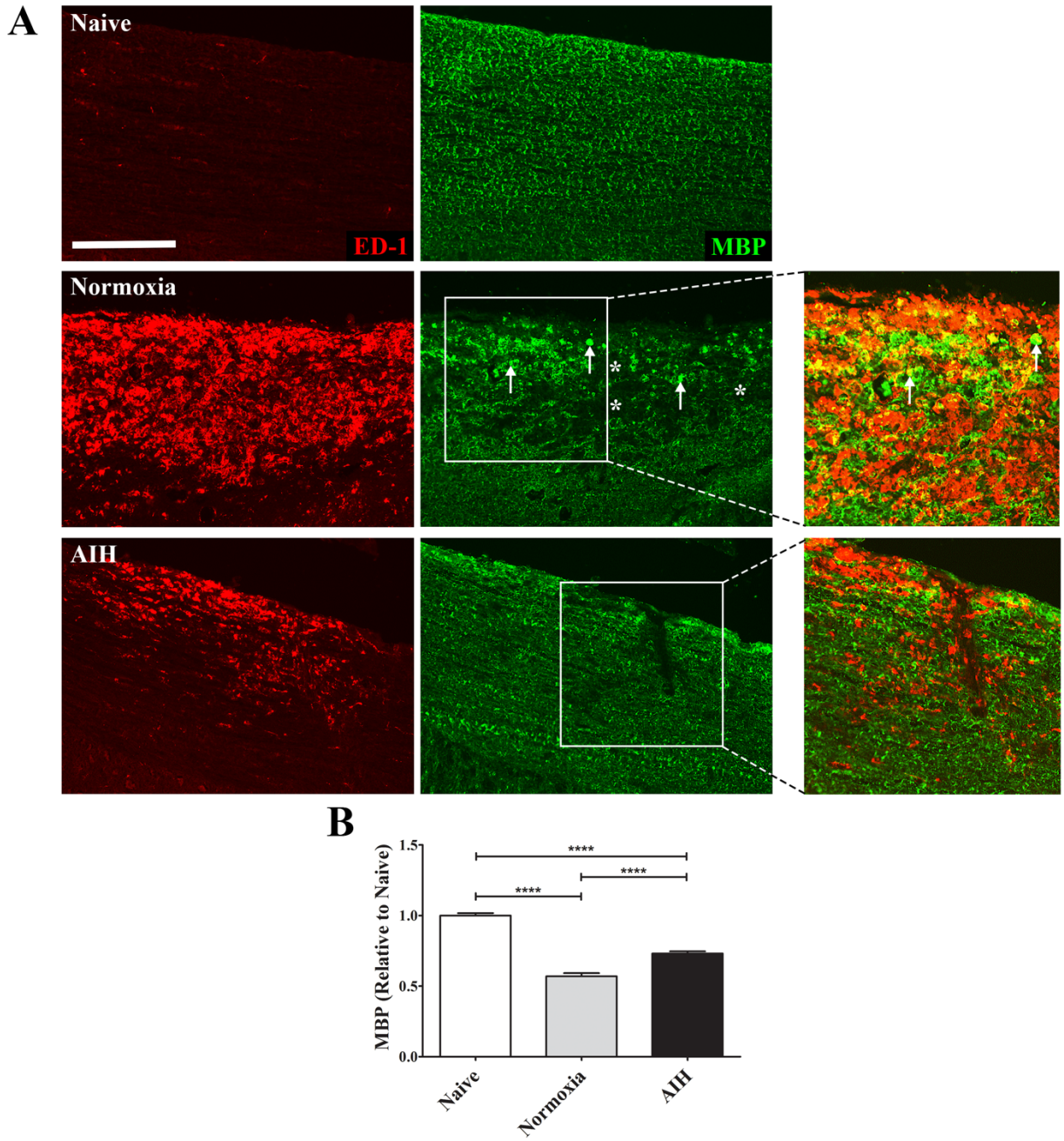

Figure 14. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a dramatic impact on levels of myelin basic protein (MBP) in regions of inflammation. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and levels of myelin basic protein (MBP). 7d AIH treatment results in a smaller inflamed area with evidence of significantly higher levels of MBP/myelin detected at 7d post last treatment in inflamed ROI as compared to Normoxia control where there

is a great loss of myelin (white asterisks). **B.** Quantification of MBP IF levels in inflamed ROI (minus MBP localized to macrophages/microglia; i.e. white arrows) in experimental tissue as normalized to MBP levels in Naïve tissue confirms qualitative observations. N=7 mice/treatment with 41-60 ROI sampled per condition; mean +/- s.e.m.; ANOVA (****$p<0.0001$). Scale bar = 200 μm.

3.3.4 AIH enhances OPC recruitment

It has been suggested that failure of remyelination in CNS diseases may be due to failure of OPC recruitment and differentiation. OPCs serve an important role in the damaged CNS by differentiating into mature oligodendrocytes in areas of demyelination and injury[39].

To identify changes in OPC recruitment to areas of demyelination, I examined PDGFRα which is a marker used to strictly detect OPCs[171]. Normal levels of PDGFRα expression are seen in the Naïve control lumbar spinal cord tissue. The 7-day Normoxia treatment group showed an increase in OPC recruitment to ROI (Figure 15). However, a large proportion of PDFRα+ve OPCs remained on or outside the border of the highly inflamed region. Further, there was an even greater increase in PDGFRα immunostaining following 7 days of AIH treatment. More specifically, rather than remaining on ROI borders, there was an influx/migration of PDGFRα+ve OPC recruitment directly into sites of inflammation observed in response to 7 days of AIH treatment (Figure 15). This provides evidence that 7 days of AIH treatment has the capability to not only enhance OPC numbers but to encourage OPC recruitment into areas of demyelination.

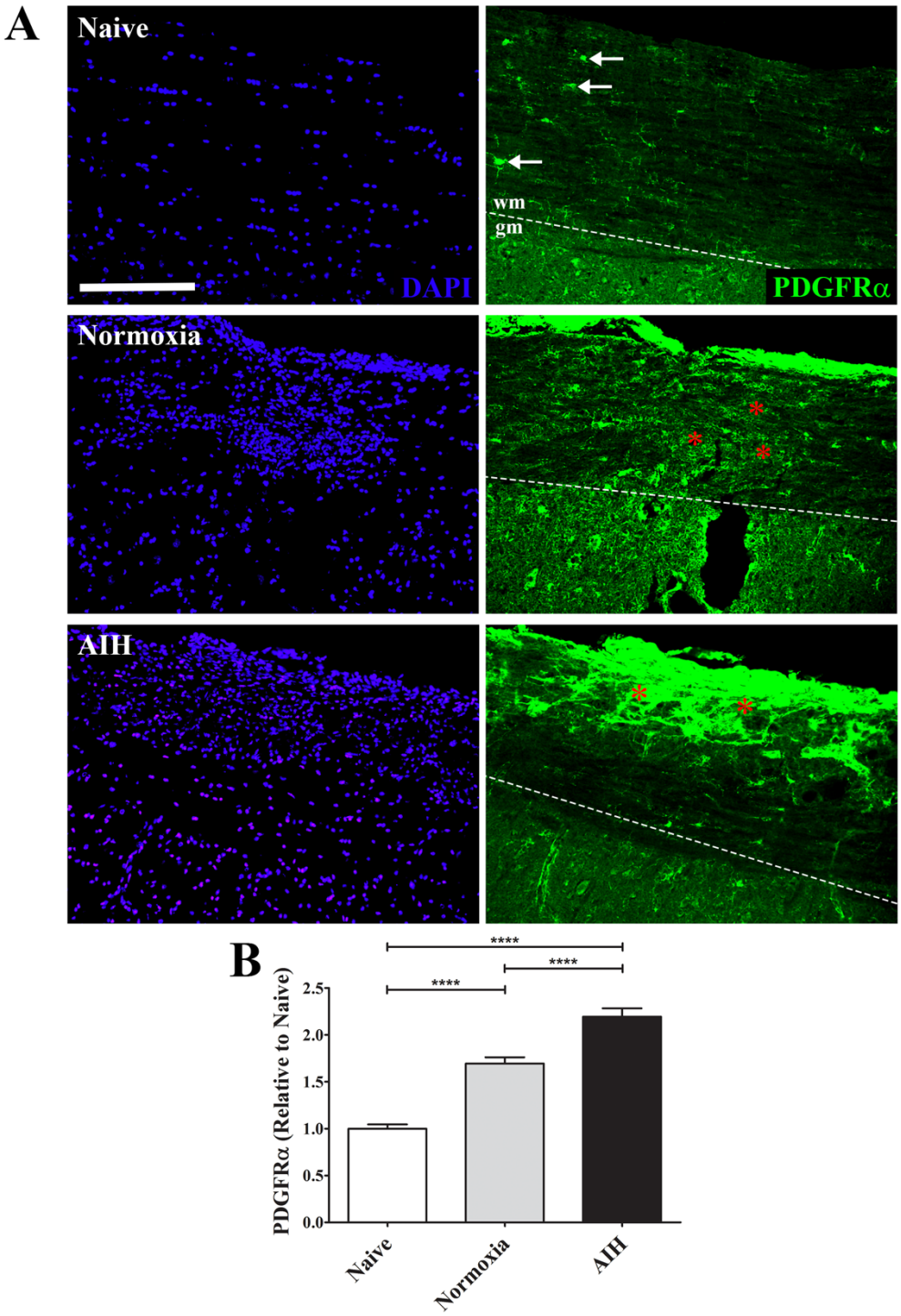

Figure 15. 7d AIH treatment started at EAE near peak of disease score of 2.5 has a marked impact on OPC recruitment. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and processed for immunofluorescence to detect oligodendrocyte precursor cells (OPCs; PDGFRα) and regions

of inflammation using DAPI in EAE mice. Note: OPCs (white arrows) are recruited to inflamed areas in EAE mice with many more OPCs recruited to sites of inflammation (red asterisks) in response to AIH relative to Normoxia controls where they tend to be found on the boundaries of the inflamed regions. **B.** Quantification of PDGFRα levels in inflamed ROI as normalized to Naïve controls confirms qualitative observations. N=7 mice/treatment with 42 ROI sampled per condition; mean +/- s.e.m.; ANOVA (****$p<0.0001$). Scale bar = 200 μm.

3.4 Impact of AIH on Immune Cell Populations

The presence of inflammation including activated macrophages/microglia and the polarization of these cells towards a pro-inflammatory or a pro-repair phenotype factor greatly in the amount of demyelination, damage and repair observed (reviewed in [172]). Macrophages/microglia are highly plastic and are able to switch between phenotypes depending on their environment. Through the examination of different macrophage/microglia IF markers, I examined whether there were changes within activated/ED-1+ve macrophage/microglia population phenotypes in response to 7 days of AIH versus 7 days of Normoxia treatment in EAE mice. All images of ventral spinal cord from the lumbar enlargement were captured under identical conditions and blinded prior to quantification to allow for unbiased analysis. In these experiments, the Naïve mouse tissue was not included in the analysis as there was no inflammation/ED-1+ve cells detected.

3.4.1 AIH enhances resolution of inflammatory response

The animal model used in these experiments is recognized for its robust inflammatory response that induces the EAE demyelinating disease, akin to the immune response and demyelination seen in MS[173]. Strategies that result in a more rapid resolution of inflammation, especially if it is one that polarizes the macrophages/microglia toward a pro-repair state, are desirable for both diminishing the degree of demyelination and inducing more effective repair/remyelination (reviewed in [174]).

The EAE mice who received 7 days of Normoxia treatment at near peak of disease score of 2.5 had high levels of activated macrophages/microglia immune cells that had infiltrated the lumbar spinal cord white matter, as detected by intense ED-1 IF (Figures 13, 14, 16). There was also the presence of MBP IF within many of these ED-1+ve immune cells (white arrows in

Figure 14), suggestive of active phagocytosis/demyelination[175]. In contrast, EAE mice who received 7 days of AIH treatment at near peak of disease, had a noticeable and significant reduction in the ED-1+ve immune cells present in the white matter tracts (Figures 13, 14, 16). In addition, the foamy, myelin debris-filled macrophages/microglia observed in the Normoxia treated animals were markedly absent in the AIH treated EAE mice, a highly reproducible observation across all animals in that group. Thus, AIH treatment appears to improve the clearance of immune cells from ROI (demyelinated regions) and overall numbers of activated macrophages/microglia observed in lateral white matter tracts in lumbar spinal cord.

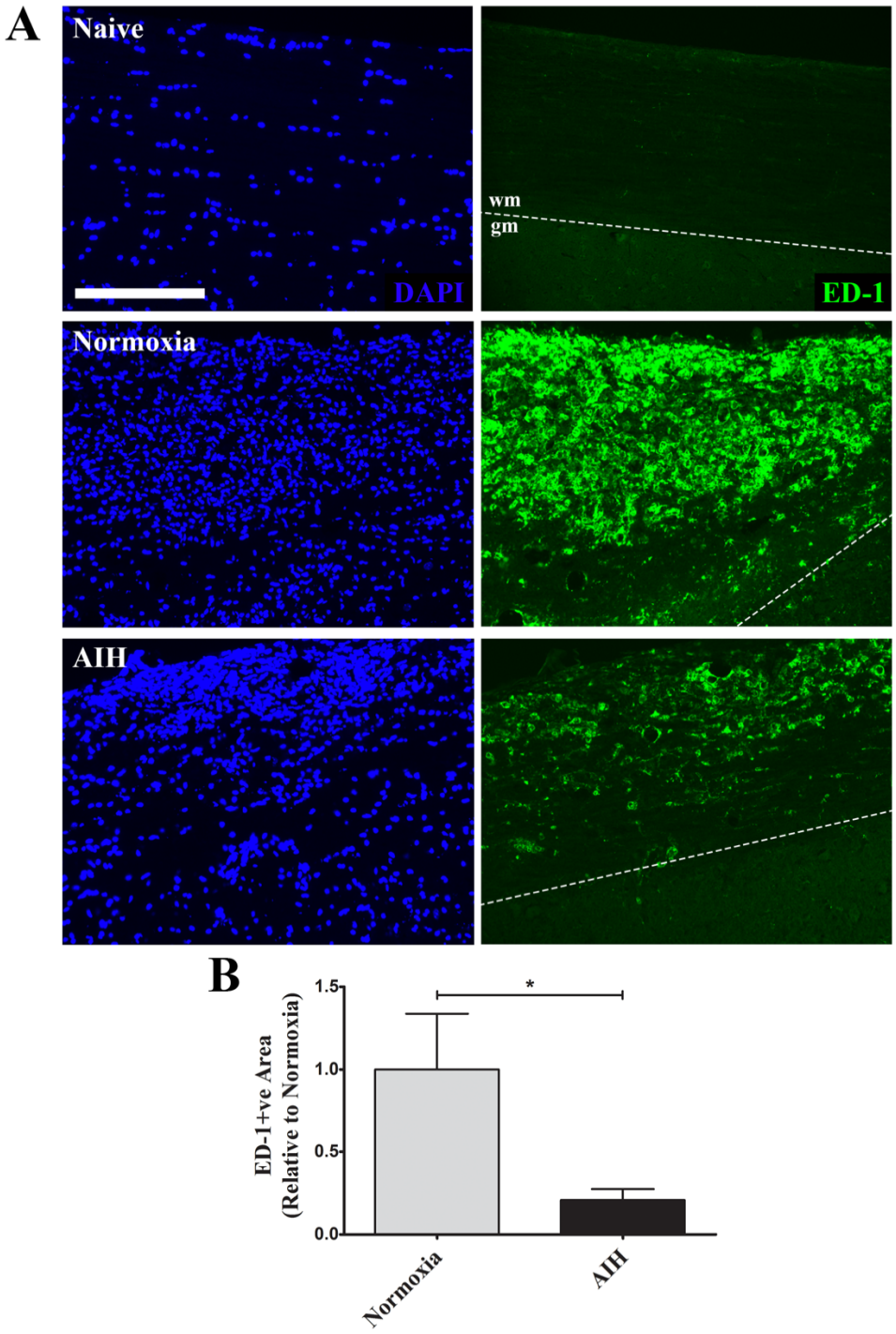

Figure 16. 7d AIH treatment started at EAE near peak of disease score of 2.5 decreases levels of activated macrophages/microglia (ED-1) in white matter. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and processed to detect activated macrophages/microglia (ED-1). 7d AIH treatment diminishes the

amount of ED-1 within lateral white matter tracts detected at 7d post last treatment compared to Normoxia suggesting that it is more resolved. **B.** Quantification of ED-1 levels within white matter tract defined ROI as normalized to Normoxia control levels confirms qualitative observations. N=7 EAE mice/treatment with 54-59 ROI sampled per condition; mean +/- s.e.m.; t-test (*$p<0.05$). Scale bar = 200 μm.

3.4.2 AIH diminishes macrophage/microglia proinflammatory phenotype

As stated previously, macrophages/microglia are highly plastic and can switch between phenotypes depending on their environment. First, I assessed M1 (proinflammatory) phenotypic markers (inducible nitric oxide synthase (iNOS) and TNFα) in the macrophages/microglia to ascertain whether AIH treatment had the potential to reduce the expression of proinflammatory molecules in these cells. Tissue was dually processed for activated macrophages/microglia marker, ED-1, and M1 IF markers. Within ROI, cells that were stained for ED-1 also expressed iNOS. In the Normoxia treatment group, in particular, there was a high degree of colocalization between ED-1+ve and iNOS IF. Comparatively, 7 days of AIH treatment resulted in a significant reduction in the level of iNOS expression in the remaining activated macrophages/microglia (Figure 17). Similar results were seen for the cytokine TNFα, another M1 phenotype marker. In the EAE mice receiving 7 days of Normoxia treatment begun at near peak of disease score of 2.5, TNFα levels within ED-1+ve cells were significantly higher than those seen in the group receiving 7 days AIH treatment (Figure 18). Therefore, the ED-1/iNOS and ED-1/TNFα colocalization data shows how AIH treatment results in both a reduction in the amount of overall immune cells as well as a reduction in the expression of M1-associated pro-inflammatory markers in those activated macrophage/microglia that remain. This supports that AIH treatment can shift immune cells away from their proinflammatory state.

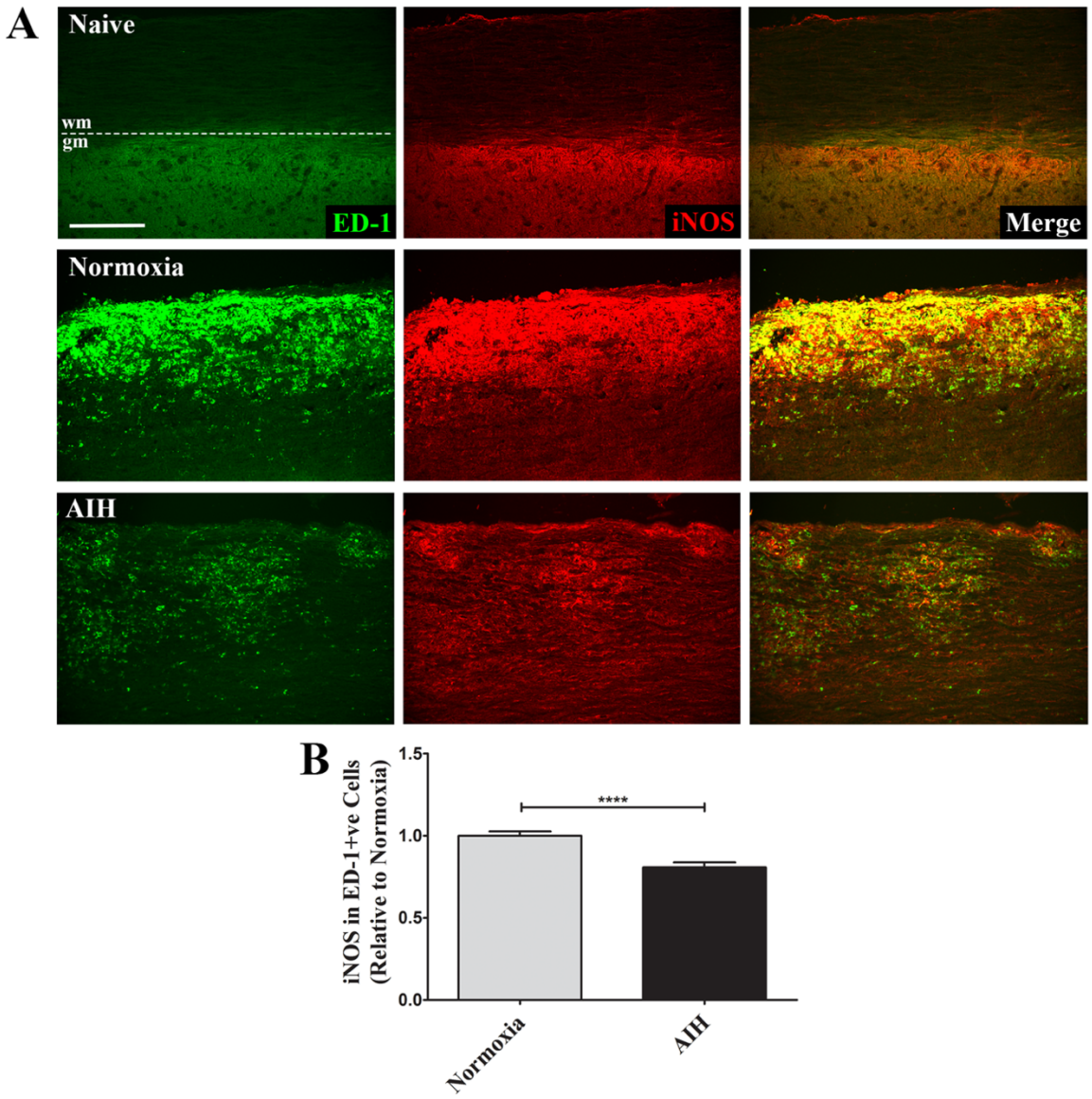

Figure 17. 7d AIH treatment started at EAE near peak of disease score of 2.5 diminishes levels of pro-inflammatory iNOS protein in activated macrophages/microglia (ED-1). A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and iNOS. 7d AIH treatment diminishes the level of iNOS detected in macrophages/microglia at 7d post last treatment compared to Normoxia. Note: the immune response appears to be more resolved in the AIH treatment groups. **B.** Quantification of iNOS levels in ED-1+ve cells as normalized to levels in Normoxia control tissue confirms qualitative observations. N=7 EAE mice/treatment with 30-51 ROI sampled per condition; mean +/- s.e.m.; t-test (****$p<0.0001$). Scale bar = 200 μm.

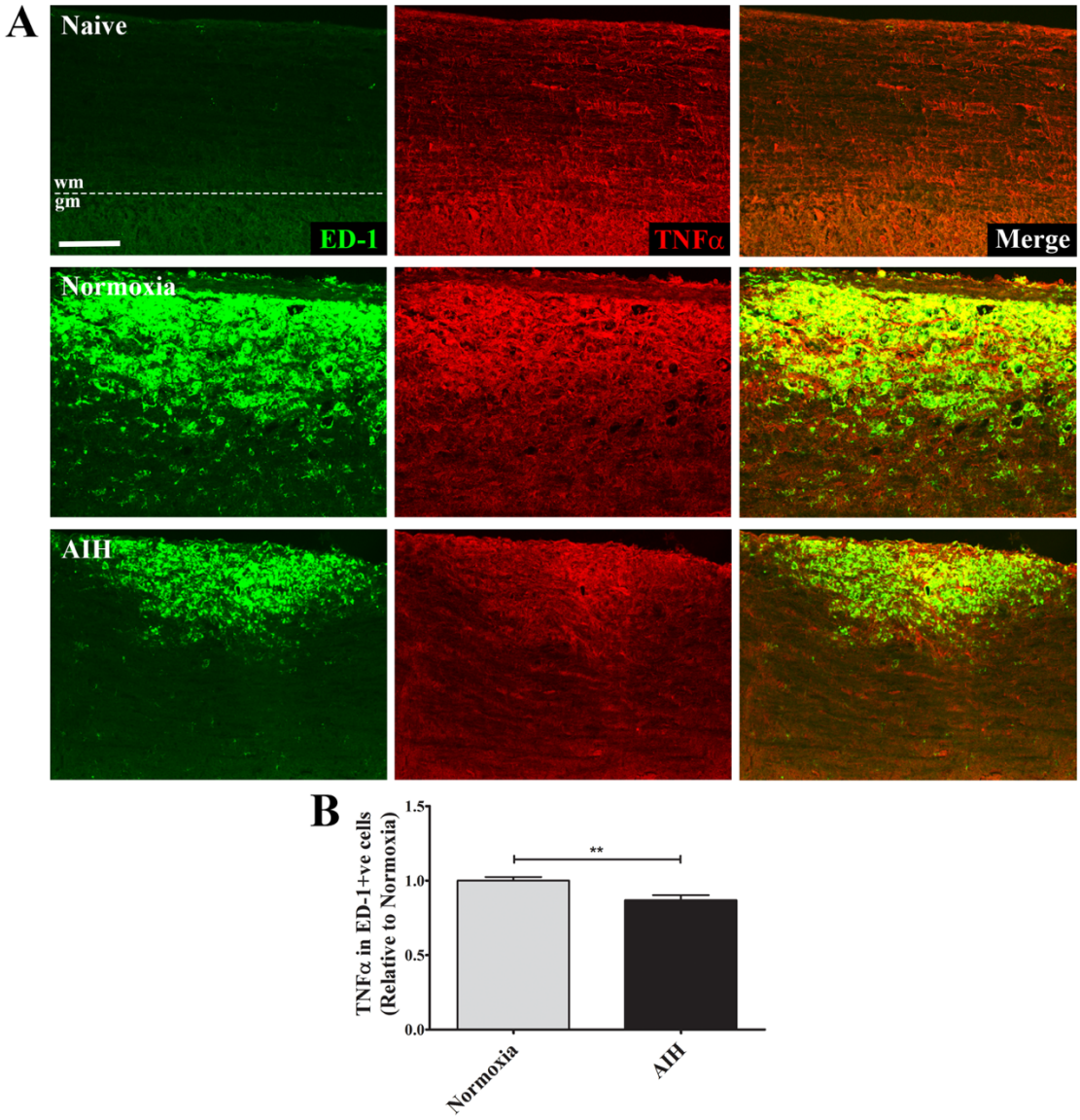

Figure 18. 7d AIH treatment started at EAE near peak of disease score of 2.5 diminishes levels of pro-inflammatory protein TNFα in activated macrophages/microglia (ED-1). A. Longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and TNFα. 7d AIH treatment diminishes the level of TNFα detected in macrophages/microglia at 7d post last treatment compared to Normoxia. Note: the immune response appears to be more resolved in the AIH treatment groups. **B.** Quantification of TNFα levels within ED-1+ve cells (ED-1 is used to create a mask within which TNFα levels are measured) as normalized to Normoxia controls confirms qualitative observations. N=7 EAE mice/treatment with 31-35 ROI sampled per condition; mean +/- s.e.m.; t-test (**p<0.01). Scale bar = 100 μm.

3.4.3 AIH enhances macrophage/microglia anti-inflammatory/pro-repair phenotype

Finally, I examined what was occurring at the opposite end of the macrophage/microglia polarization spectrum. While there were significantly higher levels of iNOS and TNFα colocalizing to ED-1+ve cells following 7 days of Normoxia, there was also a small amount of colocalization with the expression of the mannose receptor, CD206, which is found on the surface of macrophages/microglia, and the secretion and expression of activin-A protein, both of which are markers associated with the M2 pro-repair macrophage phenotype[47,176]. The macrophages/microglia in lumbar spinal cord white matter tracts in AIH-treated EAE animals contained much higher levels of both CD206 and activin-A (colocalized with ED-1) compared to the Normoxia treatment group (Figures 19, 20). Thus, the ED-1/CD206 and ED-1/activin-A co-expression data supports that the 7day AIH treatment is polarizing the macrophages/microglia toward a pro-repair M2 phenotype that is evident one week after the last treatment. Further, the heightened expression of Activin-A by these cells following AIH treatment may be helping the abundant OPCs in these areas differentiate into mature oligodendrocytes[47].

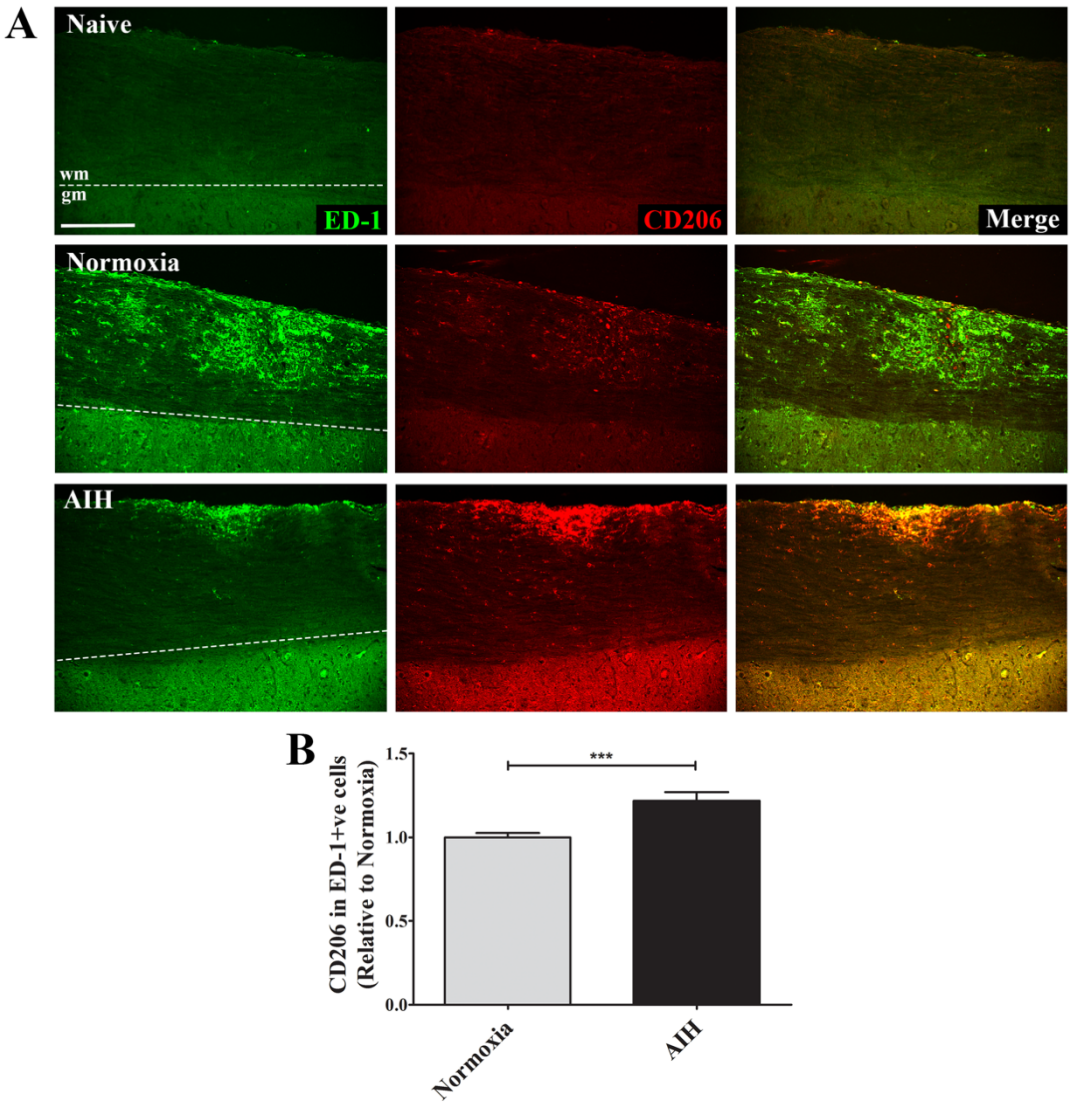

Figure 19. 7d AIH treatment started at EAE near peak of disease score of 2.5 increases levels of pro-repair molecule CD206 in activated macrophages/microglia (ED-1) in EAE mice. A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and CD206. 7d AIH treatment increases the level of CD206 detected in macrophages/microglia at 7d post last treatment compared to Normoxia. Note: the immune response appears to be more resolved in AIH treatment groups. **B.** Quantification of CD206 levels in ED-1+ve cells (ED-1 is used to create a mask within which CD206 levels are measured) confirms qualitative observations that CD206 levels are significantly increased in response to AIH as normalized to Normoxia controls. N=7 EAE mice/treatment with 28-39 ROI sampled per condition; mean +/- s.e.m.; t-test (****$p<0.0001$). Scale bar = 200 μm.

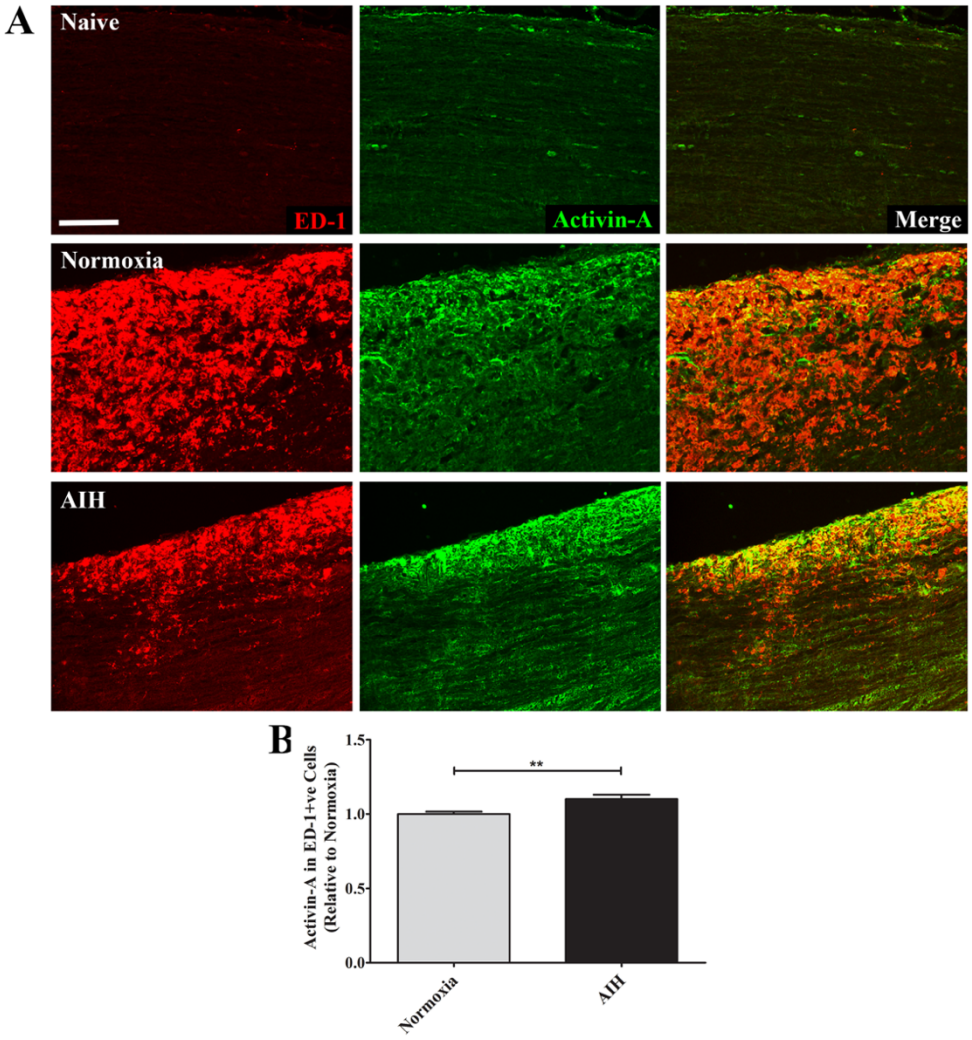

Figure 20. 7d AIH treatment started at EAE near peak of disease score of 2.5 increases levels of pro-repair molecule activin-A in activated macrophages/microglia (ED-1). A. Representative longitudinal sections of ventral spinal cord from lumbar enlargement mounted on same slide and dually processed for immunofluorescence to detect activated macrophages/microglia (ED-1) and activin-A a molecule that promotes oligodendrocyte differentiation. 7d AIH treatment increases the level of activin-A detected at 7d post last treatment in ED-1 positive cells compared to Normoxia. Note: the immune response appears to be more resolved in AIH treatment groups. **B.** Quantification confirms qualitative observations that the levels of activin-A within ED-1 positive cells (where ED-1 is used to create a mask within which activin-A levels are measured) are significantly increased in response to AIH. N=7 EAE mice/treatment with 47-50 ROI sampled per condition; mean +/- s.e.m.; t-test (**$p<0.01$). Scale bar = 100 μm.

CHAPTER 4: DISCUSSION

4.1 Summary of Major Findings

This thesis examined, for the first time, the therapeutic potential of AIH as a strategy to enhance repair of the demyelinated CNS and mitigate disease progression in the EAE mouse model of MS. The data provide evidence that one week of daily AIH treatment, when begun at near peak of disease, is an effective therapeutic strategy that targets intrinsic repair. This was in contrast with a lack of effect when the same number of treatments were begun at the first sign of disease. The beneficial effects that AIH has on EAE mice were elucidated through the use of both clinical score evaluation and histopathological investigation. Seven days of daily AIH treatment significantly improved clinical scores as compared to the Normoxia group. When examined at the tissue level, these outcomes correlated with a significant impact of AIH treatment on intrinsic repair processes in inflamed regions of spinal cord white matter, including enhanced myelination, axon protection and recruitment of OPCs to demyelinated areas. Additionally, AIH affected an overall reduction in inflammation and polarization of the remaining macrophages/microglia towards a pro-repair state. The exact mechanisms responsible for this heightened capacity for intrinsic repair are unknown. However, AIH treatment of Naïve mice produced an upregulation of plasticity-associated proteins within spinal cord tissue described in AIH studies for other pathologies and linked to the favourable outcomes observed for these studies. Collectively, these data offer novel insight into the therapeutic potential for AIH treatment in demyelinating disease and suggest mechanisms that may underlie the beneficial effects associated with it.

4.2 AIH Improves Clinical Scores When Initiated at Near Peak EAE Disease

The model employed in my studies is the monophasic acute progressive MOG_{35-55} EAE mouse model of MS which induces an ascending paralysis[177,178]. $MOG_{35–55}$ immunized mice show clinical and pathological similarities to MS in humans including CNS inflammation, focal demyelination, axonal and neuronal degeneration and gliosis[143,177]. Even though the EAE model deviates from human MS, such as MS involving both CD4+ and CD8+ T cells or the EAE model using pertussis toxin to open up the BBB and promote T cell expansion and differentiation, the similarity in clinical and pathological features with MS support the use of $MOG_{35–55}$ EAE as a model of MS[179–183]. In my thesis, the MOG_{35-55} EAE model was used to examine whether the

exposure of these mice to AIH treatment would lead to improved clinical scores and pathohistological outcomes and whether the timing of when treatment began altered this response – i.e. could AIH impact the worsening of disease and/or reverse disease?

It was found that when 7 days of once daily AIH treatment was started at the onset of EAE disease, there were no significant differences between the Normoxia control and AIH treatment groups. However, when this same treatment was started at near peak EAE disease, AIH treated animals had a remarkable, significant and sustained improvement in clinical scores compared to their Normoxic counterparts. Further, this correlated with histopathologic indicators of myelin repair, axon protection and immune response resolution. These results indicate that while AIH may not have an effect on the initial progression of EAE disease, it does have an effect on the reversal of EAE disease and promotion of repair.

The improvements observed when AIH treatment was begun at near peak of disease closely mimic the clinical scenario of the MS patient seeking treatment once an MS attack has occurred, rather than seeking treatment in anticipation of an attack. Thus, the administration of AIH at near peak EAE disease may be a more clinically relevant timepoint. Due to time restraints imposed by the COVID-19 shutdown, I was unable to look extensively at the histopathology of the first group that started AIH treatment at onset of EAE disease. This will be a future project where we will attempt to gain insight into why AIH treatment did not significantly attenuate the progression of these clinical scores.

When treatment was begun at near peak of disease, the Normoxia EAE group followed the typical EAE disease progression associated with the MOG_{35-55} EAE MS model. They reached peak of disease several days following onset, followed by a very slight recovery that was not large enough to be classified as a "remission", and then a worsening of disease back to initial near peak of disease clinical scores[184]. This differed dramatically from the AIH group, which showed a long-lasting improvement in clinical scores nearly back to normal, evident as early as two days post initiation of treatment. There was however a nonsignificant trend in increased clinical score in the last 4 days of scoring, 4 to 7 days after the last AIH treatment that may signify that the impacts of AIH are not permanent and may require additional periodic treatments to sustain the response when layered against the chronic disease platform of this EAE model. This observation is consistent with the transient but significant improvements observed in

incomplete spinal cord injury patients, that could be significantly prolonged with 3 weeks of 3 days/week additional treatments[152].

Future investigations could address this through two experiments. First, a longer experimental timeline could be used in which animals can be followed for longer than the 7 days post-last treatment to assess whether AIH animals would stop worsening or show a second recovery following worsening. In a second experimental design, EAE animals would be administered additional boosters of AIH treatments (i.e. 7 days of daily AIH treatment followed by once daily AIH treatments every third day thereafter) to see if additional treatments could repress the worsening of disease and maintain a clinical score near 0. Finally, it would also be relevant to examine whether AIH treatment effects a more rapid transition to a remission phase in the $PLP_{139-151}$ relapsing-remitting EAE model in SJL mice and whether it could be used prophylactically to prevent future relapses[173,178,185].

4.3 Intrinsic Repair Processes are Enhanced by AIH Treatment in Demyelinated CNS

The finding that AIH treatment induces efficient repair in EAE mice through the enhancement of intrinsic repair processes was arrived at by examining multiple axes of repair, including whether AIH treatment affected axon protection, node of Ranvier reorganization, myelination, OPC recruitment and immune response modulation, which it did (see discussion below). The exact mechanisms responsible for these positive outcomes are unknown, but likely involve the ability of AIH to induce plasticity[152,186,187] and elevate expression of molecules known to promote nervous system plasticity[157] – i.e. BDNF and HIF1α, which have been shown to promote repair and plasticity in the nervous system[119,135,188] and, the former, critical for inducing LTF[157].

Preliminary experiments were done that confirmed that the mice used in our study showed increased levels of plasticity-associated genes, including BDNF, GAP43 and HIF1α in lumbar spinal cord, 18 hours after 4 days of daily AIH treatment. However, due to COVID-19 delays, I was unable to generate a group of EAE animals that were to be sacrificed shortly after their last treatment to examine whether heightened expression of these genes correlated with the improved clinical scores and indicators of repair observed one week later. When plasticity-associated gene expression was examined in the tissue from EAE mice 7 days after the last AIH treatment, levels were not discernibly different from the Normoxia group, as these inductive

signals are likely no longer at heightened levels (data not shown). This is in agreement with our past studies that state that inductive signals associated with heightened plasticity responses are typically transient due to the fact that the stimulus (AIH or ES) that induced them are also transient. In AIH spinal cord injury or peripheral nerve regeneration studies where this gene expression was examined within days of the last treatment, expression of BDNF, GAP43 and HIF1a were significantly elevated[153,189], whereas if examined at later timepoints, the expression had returned to levels comparable to the Normoxia-treated animals. Similar outcomes were observed in our nerve repair studies examining the impacts of ES on nerve injury and repair, with transient rises in BDNF and GAP43 expression observed in the first few days in the ES animals but not at later timepoints relative to nerve injury and repair alone[190]. Our lab then went on to show that even the transient rise in BDNF that is observed in response to injury discharge is critical for sensory neurons to induce a regenerating state but is not required to maintain it, once again highlighting the importance of BDNF as an inductive signal for repair, similar to its critical and inductive role in promoting AIH-associated plasticity including LTF[188,191]. Thus, one must examine these inductive signals within days of treatment as they are typically not discernible a week or several weeks later.

4.3.1 AIH promotes an axon protective phenotype

Neurofilament phosphorylation is an important determinant of axonal integrity. In particular, phosphorylation of neurofilaments seems to be responsible for the prevention of axon degradation and increased axonal caliber[192]. Further, a number of studies have shown evidence of axonal degeneration being linked to dephosphorylation following demyelinating events[62,100]. Because of this, rephosphorylation of neurofilaments following demyelination or damage is important for the maintenance and protection of axons until they are remyelinated and also serves to increase the diameter of the axon, a critical signal in initiation of myelination[56,62,65]. The ability of AIH treatment to significantly elevate levels of neurofilament phosphorylation in axons within zones of inflammation in white matter tracts contrasted with the Normoxia group where there was a significant loss of phosphorylated neurofilaments within the qualitatively fewer axons that were still present with a much more disorganized appearance within the inflamed demyelinated regions suggestive of the diffuse axon injury in MS[66]. The disorganized appearance of axons in these zones of demyelination are consistent with those described for white matter tracts or

nerves[117,193]. While neurofilament phosphorylation can confer a protected state on the axons by protecting it from damage due to calpains, it is not the only cause of the neuronal and axonal degeneration associated with MS and EAE[64,65]. Other neurodegenerative events that can impact the health of neurons and axons in demyelinating disease include different stressors such as dysfunctional RNA binding protein biology that promotes stress granule persistence, mitochondrial injury/dysfunction that induces impaired energy production and increased iron deposition that leads to iron-induced oxidative stress[194–196]. While we do not yet know if AIH treatment can alter these other drivers of neurodegeneration, it warrants investigation. Preventing the permanent clinical disability associated with axonal and neuronal injury in MS is of great importance.

4.3.2 AIH promotes changes consistent with remyelination

When demyelinated axons are remyelinated, the nodes of Ranvier must be re-organized to ensure proper conductance of axon potentials[37]. Node of Ranvier organization is complex with Na^+ sodium channels at the node, contactin-associated protein (Caspr) found in the paranode region and voltage gated K^+ potassium channels in the juxtaparanode[197]. Therefore, it is possible to use markers of membrane proteins like Caspr as indicators of normal myelination. Caspr expression in demyelinated regions of spinal cord tends to present in low numbers or is absent entirely, assuming a dispersed or diffuse distribution[198]. Caspr is known to be re-expressed and re-directed during remyelination to the paranode region, which is believed to signify new, mature myelin in areas that were previously demyelinated[170].

Following induction of EAE and Normoxia treatment, paranodal node of Ranvier protein, Caspr, assumed a very diffuse or even absent distribution in inflamed regions lacking myelin as indicated by the absence of MBP. In contrast, one week of daily AIH treatment promoted the reappearance and reorganization of nodes of Ranvier as seen with a corresponding increase in Caspr within ROI that had intense inflammation. Higher numbers of paired paranodes suggest that AIH treatment promotes a phenotype consistent with remyelination. However, while Caspr+ve nodes are visible in AIH animals, the pairs of nodes, while punctate, are not as restricted as those seen in the Naïve control group. This may be because repair and remyelination are not yet complete. Thus, examining this phenotype at later timepoints may reveal more restricted Caspr+ve paranodes. Additionally, we could have examined the other aforementioned

markers of other regions of the nodes of Ranvier. However, the shift that was also seen in clinical scores, OPC recruitment (see below), activin-A expression (see below), neurofilament phosphorylation and MBP levels collectively supports that successful remyelination has occurred and/or is well underway.

4.3.3 AIH promotes a myelinated state through OPC recruitment and expression of molecules linked to myelination

As mentioned above, one potential mechanism by which AIH has been shown to impact neuronal activity is as described by its ability to promote LTF in phrenic motor neurons, an event that leads to elevated BDNF expression and is critically dependent on the de novo synthesis of this neurotrophin[157,158]. Previous research has shown that there is a strong link between neural activity and myelination of the CNS. In particular, there is evidence that activity has an important role in the regulation of oligodendrocytes which go on to myelinate axons (reviewed in [199–201]). Increased neuronal activity has been shown to increase levels of myelination in axons in the CNS compared to those that were not stimulated[202]. As such, therapies that are able to increase neuronal activity, such as AIH, thereby increasing molecular cues such as the release plasticity-associated factors (i.e. BDNF, HIF1α) that induce oligodendrogenesis and remyelination may be an effective way to improve disease outcomes[189,203–205].

Previous work in our lab has identified increased neural activity as a factor of enhanced myelin formation *in vivo* following demyelination, but this is the only known research that focuses on the impact of neuronal activity on the peripheral nervous system *in vivo* rather than *in vitro* with respect to promotion of myelination and node of Ranvier organization[117]. The data presented in this thesis enhances our understanding of neural activity and its effects on myelination and oligodendrocyte recruitment, specifically in the CNS. In the EAE animal model, demyelination of axons typically occurs shortly after onset of disease with a very slight amount of remyelination, similar to MS[139,206].

I found that AIH treatment promoted greater myelination in inflamed ROI in EAE animals as compared to the Normoxia group with higher levels of linearized MBP and a significantly higher density of Caspr+ve paranodes. In Normoxia EAE animals, high levels of MBP IF signal were localized to macrophages/microglia, suggesting that myelin debris was still being cleared in these animals. That is why it was necessary to subtract the MBP detected in

macrophages/microglia from the total MBP signal detected in the inflamed ROI in order to get a true reflection of how much MBP was likely associated with myelin surrounding axons. Unfortunately, due to the methodology employed, it cannot be said whether the axons were actually remyelinated or if they were prevented from further demyelination compared to the Normoxic controls. However, as stated previously, evidence from the rest of the presented data supports that the myelination that we are seeing is likely remyelination as I observed beneficial effects in clinical scores, the reorganization of paranodes, neurofilament phosphorylation and OPC recruitment levels (see below) in response to AIH. Additional insight will be gained upon histopathological analysis of EAE tissue from mice that have reached near peak of disease score so that the state of demyelination and inflammation can be assessed prior to initiation of treatment.

In order for remyelination to occur, OPCs must be recruited to the region of demyelination and driven to differentiate into mature oligodendrocytes[207]. When OPCs are not recruited to the appropriate areas and do not differentiate, proper remyelination to replace damaged myelin cannot occur. Not only do mature oligodendrocytes remyelinate the CNS, they also provide metabolic support to axons which if inhibited may promote additional damage to axons[42]. Therefore, improved OPC recruitment to inflamed ROI should enhance remyelination of those demyelinated regions[43,208].

I examined changes to PDGFRα+ve OPC recruitment within inflamed ROI and found that following Normoxia treatment, there is only a small increase in OPC recruitment and many of the OPCs remain outside of the ROI. On the other hand, AIH treatment resulted in a significantly higher number of OPCs and more specifically, OPCs directly within areas of inflammation. While we did not look at mature oligodendrocytes, the great recovery of function coupled with increased levels of linear MBP IF and paranodal reorganization, along with the impact that AIH has on the rest of the intrinsic repair processes, indicates that remyelination is likely occurring. Additionally, I investigated pro-repair macrophage/microglia marker, activin-A, which beyond being a phenotypic marker of immune cells that have polarized to a pro-repair state, has also been identified as a driver of OPC differentiation[47]. The AIH treatment group showed increased levels of this marker, providing further evidence that AIH influences OPCs, oligodendrocytes and myelination. More recently, Yuen et al.[203] have linked intrinsic OPC

HIF1α, a transcription factor induced by AIH to axon integrity and the onset of myelination in forebrain, further supporting the potential for AIH to promote myelination[135,153,209].

4.4 Impact of AIH on Immune Cell Populations

4.4.1 AIH drives resolution of inflammation

One of the most notable impacts of AIH in the EAE model was on the degree of macrophage/microgliosis resolution observed. Levels of inflammation influence CNS injury and repair outcomes in EAE with resolution of inflammation associated with more effective repair[210]. Further, the functional phenotype of macrophages and microglia are also closely linked to what is occurring in the tissue. The complex macrophage/microglia spectrum can be simplified into two phenotypes that correlate changes in inflammation and repair states. M1 macrophages are known to show high levels of phagocytic activity and produce and secrete proinflammatory cytokines[75–77]. It is important that the myelin debris be cleared in order for myelination to proceed[211,212]. In general, M1 macrophages are involved in the induction of the acute phase of inflammation and are associated with pathogen death and debris phagocytosis in early "healing" stages[76,85]. On the opposite side are the M2 macrophages which promote the expression of anti-inflammatory molecules. They are often associated with homeostasis, repair processes and the clearance of apoptotic cells[76,78]. Because macrophages and microglia are involved in both demyelination and remyelination, the correct balance is required to optimize remyelination and repair of the damaged nervous system. Studies that examine CNS diseases/injuries and macrophage polarization have shown that the induction of an M2 macrophage phenotype following CNS damage promotes repair responses[213–217].

The data presented in this thesis strongly supports that AIH treatment has a tremendous capacity to enhance a number of repair processes. AIH also seems to induce a quicker resolution of the inflammatory immune response that is involved with EAE disease. EAE mice that received AIH treatment instead of Normoxia treatment showed significantly fewer activated macrophages/microglia within ROI 7 days after the last treatment. There also seemed to be much less evidence of the "foamy" appearance that accompanies active phagocytic immune cells that contain myelin debris which can be indicative of the earlier and quicker resolution of myelin debris phagocytosis[210]. Due to time restraints, I was unable to determine original levels of

infiltrating macrophages/microglia, but I predict that EAE animals at near peak disease prior to treatment will show high levels of initial infiltration. This would demonstrate that AIH treatment does provide effective resolution of the immune response and thus, faster myelin debris clean-up which is necessary for consequent remyelination and repair. Levels of inflammation are also correlated with the degree of axonal damage and rapid resolution of inflammation has been linked to better axonal protection and health[100,104]. Further, a recent study by Agosto-Marlin et al.[218] showed that LPS-induced systemic inflammation can attenuate the ability to induce LTF/increased neural activity in motor neurons, one of the main mechanisms by which AIH positively impacts plasticity[219]. This is another reason why faster resolution of inflammation is desirable.

Not only does AIH treatment have a remarkable impact on the resolution of inflammation, the data presented also shows that it effects polarization of immune cells. We did not determine the phenotypic states that macrophages/microglia were in at initial infiltration of the demyelinated regions. However, comparison of the remaining immune cells between the treatment groups showed that AIH treatment influenced the immune response toward a favorable state. Following AIH treatment, the majority of immune cells were polarized toward M2 pro-repair state and expressed markers associated with this phenotype (CD206 and activin-A). Conversely, macrophages/microglia within areas of high inflammation in EAE animals subject to Normoxia control treatment were polarized primarily toward an M1 pro-inflammatory state with the majority expressing markers associated with the M1 phenotype (iNOS and TNFα).

There is evidence that macrophage phenotypes are dependent on the levels of myelin debris and phagocytosis, with higher levels of phagocytosis encouraging a switch in phenotype toward the pro-inflammatory M1 state[84]. I found that the phagocytosis of myelin debris by pro-inflammatory macrophages was still evident two weeks post near peak of disease in the Normoxia EAE mice. This has been shown to be part of the signal that leads to the production of molecules such as prostaglandin, E2, and the chemokine, CCL18, that help drive the macrophages/microglia toward an M2 pro-repair state[220,221]. Thus, there is likely a relationship between AIH's ability to resolve inflammation and induce rapid phagocytosis of myelin debris and the switch toward a pro-repair phenotype that we saw. This shift is also in parallel with studies that have shown that macrophages and microglia phagocytose even more myelin when they are in an M2 polarized state[222]. Further, previous studies within our lab have provided

evidence that electrically active axons can provide macrophages with a signal that can push a shift in polarization toward a pro-repair state[118]. This increase in neuronal activity may also be the mechanism that allows AIH treatment to induce similar results in the demyelinated CNS.

As mentioned before, pro-repair marker, activin-A, expressed by M2 polarized macrophages/microglia, is known to promote OPC differentiation into mature oligodendrocytes which is necessary for myelination to occur[47]. The increase in AIH-induced activin-A expression by macrophages/microglia coupled with the significant impact on migration of PDGFRa+ve OPCs into the same regions of inflammation provide a strong microenvironment that should encourage the differentiation of OPCs into oligodendrocytes capable of remyelinating the axons. The results presented in this thesis offer evidence for the beneficial effects of AIH treatment encouraging a crucial shift towards the pro-repair M2 macrophage/microglia phenotype that can induce repair and create an environment for optimal remyelination.

4.5 Implications of Findings

4.5.1 AIH as a potential therapeutic strategy for MS

While the main aim of this research was to determine if AIH therapy was able to enhance repair within EAE, a preclinical animal model of MS, the ultimate goal of this project is to provide the biomedical evidence that warrants exploring it as a therapeutic strategy that could be translated to clinical use. MS is understood to have multiple origins and because of this heterogeneity, it makes it much more challenging to design therapies[4,95]. Most conventional therapies currently used to treat MS focus primarily on the modulation of the immune response and affect each patient differently, perhaps due to this heterogeneity[13]. While these therapies can alleviate symptoms and mitigate damage, they rarely target repair responses associated with remyelination and repair of the damaged CNS that unless achieved, often leads to irreparable loss of neurons, axons and function in both humans and the EAE model (reviewed in [223,224]). Thus, efficient repair, prevention of axonal damage and remyelination remain a major challenge of MS.

Prior research in our lab has shown that the use of activity-dependent ES can enhance these intrinsic repair mechanisms of the peripheral nervous system following lypophosphatidyl choline (LPC) focal demyelination, which has also been recently replicated in the focally demyelinated CNS[117,225]. However, while ES of axons within a focally demyelinated pathway or

nerve is an effective therapy, it is invasive. Further, while the LPC-induced focal demyelination model is insightful for examining remyelination, it does not recapitulate many of the immune-related aspects of MS as well as other models. Therefore, by using ES as a proof of principle, investigation into non-invasive AIH treatment, which is also linked to increased neural activity, in an animal model that better approximates MS, seemed like the next logical step toward clinical relevance.

By all parameters assessed thus far, AIH appears to be a safe and effective therapy. There is evidence that AIH does not induce deleterious cognitive effects in humans as it has been demonstrated that specific treatment regimens do not elicit any visual or verbal memory impairment indicating that translation of this therapy to clinical trials is possible[226]. Additionally, AIH is already in multiple long-term clinical trials for spinal cord injuries, amyotrophic lateral sclerosis (ALS) and MS (ClinicalTrials.gov #NCT03071393, #NCT03774043, #NCT04280484, #NCT03262766, #NCT02274116). However, the small pilot study on MS, unlike the others, is being done in the absence of any pre-clinical data. The findings in this dissertation, observed both by clinical scores and histopathologically, provide data that supports the potential of AIH treatment as a viable therapy that can induce repair within the damaged and demyelinated CNS, and hopefully in MS. Further, the fact that AIH therapy showed improvement of clinical scores in the EAE mice following treatment started at near peak disease makes, this a more clinically feasible treatment as this is the timepoint at which someone with MS would seek treatment following an attack.

I believe that AIH treatment could also be used as an adjunct therapy in combination with conventional treatment, as spinal cord studies have shown that when coupled with task specific training, the impacts of AIH are increased in both animals and humans[152–154,227]. More specifically, exercise has been found to be a beneficial intervention in improving impairments in MS patients[228,229]. However, for patients with severe cases of MS such as those who have lost limb function, independent exercise is impossible. Thus, it is excellent that in this thesis, AIH was able to effect its positive outcomes without additional training. That being said, a lot of the beneficial effects seen with exercise are thought to be induced by the increase in neural activity and consequent enhanced plasticity[230]. Thus, theoretically exercise and AIH treatment should produce synergistic or perhaps additive outcomes and is an avenue worthy of exploration when possible.

4.5.2 Future directions

The main aim of this thesis was to investigate if AIH treatment had the potential to improve repair within the demyelinated CNS. The data presented allow us to conclude that AIH treatment does enhance multiple intrinsic repair processes following CNS demyelination and therefore, because of its non-invasive nature, is a potential therapeutic strategy to induce repair and remyelination in CNS demyelinating diseases such as MS. The results obtained in this thesis contribute to a larger body of knowledge about AIH treatment in nervous system repair and also give rise to new research questions and directions to be investigated.

The interruption of ongoing experiments due to COVID-19 meant that some results intended for inclusion in the thesis will now have to be completed at a future date. These include: (i) the verification of IF findings by western blot analysis of tissues already collected. While this data will hopefully confirm the histopathological findings, they will lack the cellular and potential mechanistic insights achieved through the approaches employed in this thesis; and (ii) I have also collected spinal cord tissue from EAE mice at the near peak of disease score of 2.5 to ascertain the histopathology before the start of Normoxia or AIH treatment. This would allow us to better understand exactly how much mice were improving or worsening, histopathologically, rather than just looking at AIH effects in comparison to Naïve and Normoxia controls 7 days after the last treatment.

Further, as stated previously, I would like to explore the impact that additional AIH treatments might have on clinical score outcomes. I observed a slight non-significant worsening of EAE disease that began 4 days post-last AIH treatment. But, by giving additional doses of AIH treatment, maybe there would be a second improvement in clinical scores which could potentially correlate with even better repair. It would also be interesting to identify if, with additional treatments, disease could be maintained at a steady clinical level for longer periods of time. In support of this, Navarrete-Opazo et al.[152] did show that for incomplete spinal cord injured patients, 5 days of AIH therapy coupled with locomotor training improved walking speed by 82% and walking endurance by 86% with additional exposures significantly prolonging these favourable outcomes.

While the data presented here and previous work suggest that AIH treatment evokes its beneficial effects due to increased neuronal activity, we did not directly investigate this in this study[155,231]. To provide more insight into this AIH mechanism of action, we could explore

markers of increased neural activity immediately following AIH treatment to see if their levels would be inflated within neurons. For example, one marker that could be used is the immediate early gene, *c-fos*, which has been used for many years and in many studies as an indicator of neuronal activity[232]. To further elucidate AIH mechanisms, additional exploration into differences between Normoxia and AIH treatment groups should be conducted. In particular, differences in gene regulation between these two groups could provide a more accurate understanding of what is happening on a cellular level and identify new therapeutic targets. To do so, we could use RNA sequencing (RNA-seq) to determine exactly what genes are turned on or off and what their corresponding expression levels are in each treatment group.

In conclusion, the findings of this thesis provide the first evidence for the use of AIH treatment as a novel, promising therapeutic strategy that can enhance repair in demyelinating CNS diseases like MS. Future investigations that can confirm these results and provide additional insight into the mechanisms and benefits of AIH treatment will allow for the translation of this research into clinical studies.

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