Antigenic induction of nerve growth factor (NGF) in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS)

by

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For those with multiple sclerosis

May They Learn to

Live & Laugh

With Multiple Sclerosis

And for Rita Levi-Montalcini
(1909-2012)

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ABBREVIATIONS

AC: Active control

ANOVA: Analysis of variance

ATP: Adenosine -5'-triphosphate

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

CNS: Central nervous system

CSF: Cerebrospinal fluid

CX3CL1: Fractalkine

CX3CR1: Fractalkine receptor

DRG: Dorsal root ganglia

DMT: Disease modifying therapies

DPI: Days post induction

EAE: Experimental autoimmune encephalomyelitis

ELISA: Enzyme linked immunosorbent assay

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IHC: Immunohistochemistry

IFN-γ: Interferon –γ

IL: Interleukin

MAG: Myelin associated glycoprotein

MBP: Myelin basic protein

MeCP2: Methyl CpG binding protein 2

MHC: Major histocompatibility complex

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple Sclerosis

NC: Naïve control

NDS: Neurological disability scores

NGF: Nerve growth factor

NK: Natural killer

NPP: Neuropathic pain

OGs: Oligodendrocytes

OPs: Oligodendrocyte precursors

PLP: Proteolipid protein

PNS: Peripheral nervous system

RRMS: Relapsing remitting multiple sclerosis

SC: Spinal cord

SPMS: Secondary progressive multiple sclerosis

TEM: Transmission electron microscopy

Th: Helper T cells

TNF α: Tumor necrosis factor alpha

TNFR: TNF receptor

Trk: Tropomysin kinase receptor

WB: Western blot

qRT-PCR: Quantitative real time polymerase chain reaction

ALL FIGURES AND ILLUSTRATIONS PRESENTED IN THIS THESIS REPRESENT ORIGINAL WORK BY OUR GROUP

CHAPTER 1: GENERAL INTRODUCTION

1.1 BACKGROUND AND RATIONALE FOR THESIS

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of central nervous system (CNS) myelin affecting over 2.5 million people worldwide [1]. The hallmark demyelination associated with this disease promotes axonal degeneration which leads to the permanent disability seen in MS patients. While, there are several options for the treatment of MS they only at best help to slow disease progression and minimize the disease-induced symptoms by modifying the inflammatory events associated with this disease. As such, there is no cure for MS. There are many challenges to improving the care and quality of life of patients with MS, however, one of the most significant obstacles researchers currently face surround the issue of re-myelination of damaged segments of CNS myelin. Molecular biomarkers that facilitate early diagnosis prior to the primary demyelinating event will facilitate early treatment intervention in which may potentially reduce long-term disability. Furthermore, the identification of therapies that may enhance the repair of damaged myelin or recover the loss of myelin will be an integral step in the design of effective treatments aimed at reversing the existing myelin damage. Therefore, it is imperative that current research paving the way for new MS therapies address the critical issue of re-myelination. The next frontier of MS therapeutics will be a dual approach that involves utilizing a drug with immunomodulatory effects in conjunction with effects that foster myelin repair.

Neurotrophins are immunomodulators regularly expressed in the nervous system. The ability of these proteins to act on injured neurons, prevent and reverse neurodegeneration, stimulate axonal regeneration, modulate synaptic mechanisms, and regulate neuronal plasticity [2, 3] makes neurotrophins promising therapeutic candidates for several neurological disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amylotrophic lateral sclerosis, and MS [4, 5]. In the field of MS, research suggests the neurotrophin, nerve growth factor (NGF), may play a role in the amelioration of disease processes [6-11]. In the cerebrospinal fluid (CSF) of MS patients NGF levels are increased during acute attacks and subsequently decreased during stable phases of the disease [6, 12]. This reported increase in NGF may be critical to the recovery following an immune system-mediated insult on myelin that subsides to normal levels once the myelin has been repaired as evident during the stable form of the disease. Furthermore, NGF can ameliorate clinical symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [8, 9]. Thus, NGF appears to be beneficial in reversing the disease process, although the exact mechanism is unclear. Moreover, NGF antibodies have been shown to exacerbate neuropathological signs of EAE [10]. These studies also support the beneficial role of NGF in recovery process following an MS-induced attack. Other studies suggest NGF is an anti-inflammatory mediator that can interfere with the functions of inflammatory cells in the CNS [9]. This suggests NGF may have immunomodulatory effects that benefit the reversal of the disease process associated with MS. Most notably, NGF infusion into a demyelinated lesion enhanced re-myelination [11] which implies NGF may be involved in re-

myelinating events that promote the reversal of debilitating demyelination associated with MS.

Thus, these key findings support our interest in identifying the expression patterns of NGF in an animal model of MS. In addition, it also emphasizes the importance of studying NGF during the early inflammatory stage of EAE prior to demyelination so that we can understand how to prevent disease progression before widespread demyelination occurs. As such, early detection leads to early treatment intervention thereby minimizing myelin damage and slow disease progression [13, 14]. Furthermore, since inflammatory processes contribute to demyelination and neurodegeneration, exploring the changes in NGF expression may provide new insights into potentially reversing the existing damage to CNS myelin that contribute to the permanent neurological deficits seen in patients with MS.

1.2. PROJECT SUMMARY

In my Master's thesis, we investigated the change in gene and protein expression of NGF in a well characterized EAE animal model of MS [15-19] during the acute inflammatory phase of EAE prior to demyelination. We demonstrated that antigenic induction of EAE leads to changes in NGF gene and protein expression throughout the disease course that can be correlated to neurological disability, neurological recovery, and changes in myelin integrity. Furthermore, this study recognizes the importance of one of the predominant isoforms of NGF (25 kDa) as being biologically active. The results presented in this study supports the role of NGF in ameliorating the inflammatory processes contributing to myelin damage and provides new insights into the potential benefits of NGF as a novel therapeutic agent for MS.

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PROLOGUE TO CHAPTER 2: RATIONALE FOR THE NGF REVIEW MANUSCRIPT

This manuscript was written to provide a comprehensive review of the major aspects of MS and the potential benefits of NGF that can be explored for its treatment. This manuscript was written to reinforce concepts of our previously published work which demonstrated the importance of peripheral nervous system (PNS) activation via the dorsal root ganglia (DRG) during EAE, as well as, to introduce the concept of cytokine-neurotrophin interactions that may be involved in myelin repair. In addition, this article serves to provide a solid conceptual foundation and essential background information required for my thesis and the original research that I present in this thesis. By reviewing the literature on MS and NGF, I was able to provide evidence demonstrating the several benefits of NGF that may be advantageous in the treatment of MS. Through this I was able to establish a rationale for investigating NGF experimentally during the early stages of inflammation that contribute to myelin damage.

CHAPTER 2: REVIEW: EXPLORING THE ROLE OF NERVE GROWTH FACTOR IN MULTIPLE SCLEROSIS: IMPLICATIONS IN MYELIN REPAIR

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STATEMENT OF CONTRIBUTION

I am listed as the first author for this manuscript published July 2013. I was responsible for the inception and design of the manuscript, as well as, writing the initial draft. In collaboration with my co-authors, I performed the majority of literature searches and was involved in the preparation and revision of the final manuscript and copy proofing.

2.1. ABSTRACT

Multiple sclerosis (MS) is a chronic disease resulting from targeted destruction of central nervous system (CNS) myelin. MS is suggested to be an autoimmune disease involving the pathogenic activation of $CD4^+$ T lymphocytes by a foreign antigen in the peripheral blood. The activated $CD4^+$ T cells liberate inflammatory cytokines that facilitate the breakdown of the blood-brain barrier promoting their passage into the CNS. Inside the CNS, $CD4^+$ T cells become re-activated by myelin proteins sharing a similar structure to the foreign antigen that initially triggered the immune response. The $CD4^+$ T cells continue to liberate inflammatory cytokines such as tumor necrosis factor α which activates macrophages and antibodies responsible for the phagocytosis of myelin. Acute CNS lesions can be re-myelinated, however, the repair of chronic demyelinating lesions is limited, leading to permanent neurological deficits. Although current MS treatments reduce severity and slow disease progression, they do not directly repair damaged myelin. Henceforth, recent treatment strategies have focused on neurotrophins such as nerve growth factor (NGF) for myelin repair. NGF promotes axonal regeneration, survival, protection and differentiation of oligodendrocytes and facilitates migration and proliferation of oligodendrocyte precursors to the sites of myelin damage. NGF also directly regulates key structural proteins that comprise myelin. Interestingly, NGF also induces the production of brain-derived neurotrophic factor, another integral neurotrophin involved in myelination. The intricate signaling between neurotrophins and cytokines that

governs myelin repair supports the role of NGF as a leading therapeutic candidate in white matter disorders such as MS.

Keywords: Cytokines; Multiple Sclerosis; Myelin; Nerve Growth Factor; Neurotrophins; Oligodendrocytes; Re-myelination

2.2. INTRODUCTION

Multiple Sclerosis (MS) is a chronic progressive neurological disease of the central nervous system (CNS) that is characterized by inflammation, subsequent axonal demyelination, and axonal degeneration [1-3]. MS is thought to be an immune-mediated disease of the CNS implicating T cells, especially CD4+ T helper 1 (Th1) cells, in the pathogenesis of MS. In accordance with this autoimmune theory, other $CD4^+$ cells, such as Th2 [4] and Th17 [5-8], $CD8^+$ T cells [9-14], and B cells [15] have also been implicated in the underlying pathology of MS. Support for this immune-mediated view is recognized in part by the fact that immunization with a myelin antigen such as myelin basic protein (MBP), proteolipid lipoprotein (PLP), or myelin-associated glycoprotein (MAG), or myelin oligodendrocyte glycoprotein (MOG) [16, 17] can induce experimental autoimmune encephalomyelitis (EAE) in animals. EAE is a CD4+ Th 1 phenotype mediated inflammatory disease [18] which shares many of the clinical and pathological features of that depicted in human MS [19]. However, recent research into alternative pathological explanations for MS has created a conceptual shift away from which solely involves autoimmunity to that of axonal degeneration [20, 21]. At present,

researchers are gaining a newfound appreciation of the extent to which the neuron and its axon are affected in MS. Advancements in neurobiology in regard to axon-glial and, particularly, axon-myelin interactions demonstrate the importance of these interactions in influencing disease progression and offer novel insight as to alternative pathological mechanisms of MS [20, 22, 23]. Irrespective of these independent views on disease pathology, both immune mediated and neurodegenerative processes are likely involved in the underlying pathogenesis of MS.

Due to the lack of human MS tissue samples, MS research has largely relied on a variety of animal models of MS [24-26] to explore the intricate cellular signaling that regulates myelin structure, function and repair. Animal models using MBP as the antigen, characteristically activate lymphocytes that mediate an inflammatory response without demyelination [27]. However, the demyelination commonly associated with human MS, is displayed in animals treated with the quantitatively minor constituent protein of myelin called MOG [27]. The MOG EAE model produces consistent and reproducible patterns of disease activity during the *inflammatory - pre-demyelinating*, *demyelinating* and *re-myelinating* phases of the disease [28, 29]. Hence, the MOG mouse model of EAE is recognized as the preferred model to study MS lesion repair [17, 30, 31].

2.2.1. AUTOIMMUNE THEORY

Based on the prototypical immune-mediated view, MS results from the activation of $CD4^+$ Th1 cells in the peripheral immune system where they become pathogenic towards

myelin. Following activation, $CD4^+$ Th1 cells release inflammatory cytokines, such as tumor necrosis factor α (TNF α), interferon-γ (IFNγ), and interleukin (IL) -1, -6 and -12 [32-38]. Activated $CD4^+$ Th1 cells eventually adhere to the blood brain barrier (BBB) where they continue to liberate inflammatory cytokines [39, 40]. The continued production of inflammatory cytokines at the level of the BBB eventually weakens its structural integrity which allows $CD4^+$ Th1 cells to invade the CNS where they become reactivated in the presence of CNS myelin. Activated $CD4^+$ Th1 cells continue to produce inflammatory cytokines, which subsequently drives the targeted, immune-mediated response against CNS myelin *via* indirect activation of phagocytic cells [40-45]. As a result, oligodendrocyte progenitors (OPs) attempt to proliferate and become mobilized for subsequent recruitment to the site of injury in order to re-myelinate denuded axons [46-49]. However, they are unable to completely repair the damaged myelin. The cellular mechanisms that govern this "insufficiency" are currently unknown. As the disease progresses, the myelin coating along neuronal axons continues to erode, creating long segments of denuded axons with associated oligodendrocyte (OG) death. Although several studies have shown that OPs exist in the adult CNS [50-52] their inability to appropriately migrate to, and proliferate/differentiate at the site of injury, have only resulted in partial or incomplete myelination [46, 53, 54]. Previous studies have also targeted the effects of growth factors on OPs to stimulate re-myelination, but with only marginal success [52, 55, 56].

Acute small CNS lesions can be successfully re-myelinated [57-60], however, the continual repair of chronic demyelinating lesions is limited, resulting in irreversible

neurological deficits [2] and progressive permanent clinical disability. Although current immunomodulatory (IMA) therapies slow disease progression, decrease severity and duration of relapses, as well as, decrease burden of disease [61], none of the available treatments are directly able to re-myelinate areas of damaged myelin. As a result, MS research has recognized the importance of neurotrophins, such as nerve growth factor (NGF), as a potential novel therapeutic strategy to facilitate re-myelination of MS-induced lesions.

2.3. CYTOKINE – NEUROTROPHIN INTERACTIONS

It is known that elevated levels of the inflammatory cytokine, TNF α in the CNS correlates well with MS disease activity and BBB damage [62]. TNF α induces the expression of NGF [63, 64] and brain-derived neurotrophic factor (BDNF) in a reciprocal manner [65, 66]. The beneficial effects of BDNF on myelination are well established [67-74]. Interestingly, NGF also affects the activity of TNF α action by regulating its interactions with the TNF receptors [75]. Specifically, in the presence of reduced levels of NGF, the destructive inflammatory apoptotic effects of TNF α are facilitated via its interaction with TNFR1 receptor [76, 77]. However, as NGF levels increase, TNF α activity shifts to preferentially interact with TNFR2 to facilitate myelin repair [78]. Furthermore, NGF has also been shown to ameliorate MS symptoms by inducing the expression of BDNF in DRG and CNS [79-83]. Although evidence supports the role of NGF regulation of BDNF in terms of myelination, [67, 79, 80, 82-85] the exact mechanism of NGF's effects on BDNF expression are not well known. Thus the intricate

interconnected network of NGF, TNF α and BDNF, form a key signaling triad that utilizes the key anatomical connection between the dorsal root ganglia (DRG) and spinal cord (SC) to exert their effects on myelin repair [66, 75, 86-88] **(Figure 1a & 1b)**. Therefore, NGF is recognized as a critical factor and a novel candidate for targeted repair of MS-induced myelin damage [89].

2.4. THE STRUCTURE OF THE MYELIN SHEATH

Myelin is an electrically insulating material required for the normal physiological functioning of the nervous system. Mature OGs extend large sheets of lipid membranes that wrap around axonal processes, forming a multi-layered sheet, called myelin sheath, which covers the axon in segments called internodes. Between internodes are the nodes of Ranvier [90], which are densely packed with Na^+/K^+ ATPases and voltage-gated Na^+ and K^+ channels that are involved in the generation of action potentials or electrical nerve impulses [91]. The nodes of Ranvier are the sites of saltatory conduction where action potentials jump from one node to the other. Therefore, myelin acts to effectively increase the rate of nerve impulse propagation down the axon.

The myelin sheath is a highly organized arrangement of lipid membranes and proteins [92]. Despite decades of research, the exact mechanisms regulating the formation of myelin remain a mystery. Several genes have been identified as being critical for normal myelination at the various developmental stages of myelin formation [93]. There are numerous myelin specific proteins that make up the myelin sheath. PLP is the most

abundant of these and makes up 17% of the total protein content. MBP is the second most abundant protein (8%), followed by 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (4%). MOG and MAG make up 2% of the total protein together, with the other 69% of the proteins being made up of 16 identified, and numerous as yet unidentified proteins [93]. Each of these proteins has a different, yet essential role in myelin function.

MBP is critical for the formation of normal myelin membrane extensions [94]. It acts as an adhesive protein for the cytosolic surfaces of compact myelin forming the major dense line of the myelin sheath [95-97]. The exact function of PLP is not known, although it is known to be critical for the integrity of the axon-myelin connection with age [98]. The arrangement of MBP and PLP in the lipid bilayer of the cell membrane must be precisely balanced to ensure the correct multi-layered structure of the myelin sheaths [97]. Even small changes to this balance result in significant changes in myelin adhesion or stability, thus affecting formation and function [99]. MAG adhere the OG membrane to the axolemma at the paranodal region [100, 101]. In addition, some myelin-specific proteins act as structural proteins. CNP binds to microtubules and F-actin, promoting polymerization activity and is involved in myelin formation and maintenance throughout life [102]. The exact function of MOG has yet to be clarified. However, it has been suggested that it may act as an intermediary between the myelin sheath and the immune system [103]. Studies in transgenic animals have shown significant roles for both MBP and PLP in myelin function [104]. Reduced expression of MBP is known to cause significant defects in nerve signaling [94, 105]. The order of the OG cytoskeleton and associated CNP is dependent upon MBP organization [106]. Conversely, reduced PLP

expression appears to have little effect on the development or structure of myelin, but does affect axonopathy [107]. However, in PLP over-expressing mice, the OG interaction with neurons is impaired [108]. In the absence of PLP, myelin is physically labile and tends to delaminate. PLP-deficient mice survive for nearly two years and develop ultra-structural myelin abnormalities [104, 109, 110] and signs of axonal degeneration [111, 112]. In transgenic mice, several-fold over-expression of CNP leads to a premature differentiation of OGs, and specific defects in deposition of MBP and in myelin compaction [113]. The MAG knock-out model is another relevant mouse model of myelin deficits. Studies have shown decreased expression of MAG in Schizophrenia [114, 115]. MAG is known to interact with neuronal membranes where it helps maintain the periaxonal space of myelin sheaths [116]. It is involved in the initiation of myelination, and has been shown to inhibit neurite outgrowth and impair axonal regeneration [117]. This has led to the hypothesis that MAG promotes maturation, maintenance and survival of myelinated neurons [117]. Alterations in the expression patterns of these key proteins, as identified by gene expression changes, contribute to the dysfunction of white matter in disorders such MS [105, 111, 118, 119].

2.5. NGF MOLECULAR SIGNALING

NGF was first discovered and characterized in the 1950's by Rita Levi-Montalcini [120]. It was initially named for its ability to stimulate growth and differentiation of sympathetic neurons in the peripheral nervous system (PNS) [87, 121-123]. NGF belongs to a family of structurally related proteins called neurotrophins that includes BDNF [124],

neurotrophin-3 (NT-3), NT-4/5, NT-6, and NT-7 [125]. Like other neurotrophins, NGF is first synthesized in a pro-form. The pro-NGF exists in two sizes, 35 and 27 kDa [126-128] which are cleaved to form mature NGF, which is 13 kDa in size [127-129]. Pro-NGF demonstrates neurotrophic activity similar to mature NGF, however, it is less active [130] and signals opposite functions from its mature form, via interaction with a different receptor. There are two transmembrane receptors for NGF; the low affinity p75 neurotrophin receptor ($p75^{NTR}$) and the high affinity tropomyosin kinase receptor (Trk) A (TrkA) [131-133]. $p75^{NTR}$ is a member of the TNF receptor (TNFR) superfamily [134] and has a cytoplasmic region that contains a 'death' domain [135]. Pro-NGF has a high affinity for $p75^{NTR}$, which interacts with sortilin (a type 1 membrane receptor expression by neurons) to induce signaling pathways that lead to $p75^{NTR}$-dependent apoptosis [136-138]. Pro-NGF also binds to TrkA with low affinity [139] **(Figure 2)**. After secretion, NGF is internalized in a receptor-dependent manner through TrkA, is transported retrogradely from distal axons to the neuronal cell body [140].

2.6. NGF BIOLOGICAL FUNCTIONS

NGF plays a critical role in the development and maintenance of the CNS [141]. Specifically, NGF has been shown to possess neuro-protective [142-146], immunosuppressive [147, 148], and immunomodulatory functions [148-150]. The results of various EAE animals studies have shown that exogenous administration of NGF delayed the onset of clinical EAE and, pathologically, prevented the full development of EAE lesions [63, 89], while administration of NGF antibodies exacerbated

neuropathological signs of EAE [151]. Human MS studies also support the beneficial role of NGF. For example, cerebral spinal fluid of MS patients show increased NGF levels during acute attacks that significantly decrease during remission [152]. These results, suggest that NGF levels increase in response to the immune system insult on myelin in order to facilitate neurological recovery. Once the myelin is repaired as evident by complete neurological recovery, NGF levels subside back to baseline values required for normal myelin maintenance. The beneficial effects by which NGF promotes neurological recovery is thought to occur *via* its ability to enhance axonal regeneration [85, 153] and re-myelinate axons via its protective and survival effects on OGs and OPs [78, 154, 155]. This suggests NGF may act to induce re-myelination through activity on innate OPs. [152]. In addition, the immunomodulatory effects of NGF become evident by the fact that neurons of the CNS, microglia [156], astrocytes, OGs, immune cells, such as T cells [157, 158], B cells [159], and mast cells [160, 161] have all been identified as cellular sources and targets of NGF. Thus, there are populations of several cell types that are sources of, and can respond to NGF under normal and pathological conditions. The beneficial biological effects of NGF identified in both animal and human studies, taken together with its direct effects on the various cell types involved in myelination, poise NGF as a key mediator that regulates myelin formation and repair.

2.6.1. PROTECTING AXONAL INTEGRITY

MS has a large neurodegenerative component, which was initially thought to be secondary to primary immune-mediated processes. However, recent studies suggest

neuro-degeneration may occur at the same time as or prior to immune-mediated inflammation [20, 22, 23]. Both immune-mediated and inflammatory processes start during the earliest stages of MS [162]. In addition, axonal injury and degeneration occur during both acute inflammatory demyelination [163, 164] and chronic demyelination [165-168]. Compelling evidence supports the view that neuro-degeneration is the major cause of irreversible neurological disability seen in MS patients [169-172]. Neuro-degeneration, leads to the loss of axons, which may limit viable axonal re-myelination because axons need to be intact in order to promote myelination. NGF's neuroprotective action [142-146], coupled with its ability to enhance axonal regeneration [85, 153, 173] and promote axonal branching and elongation [174], demonstrate its importance in the integral steps associated with myelin repair [75].

2.6.2. EFFECTS ON OGs AND OPs

Myelin and myelin producing cells are important for axonal support. Signals from OGs has been shown to be sufficient to promote accumulation and organization of neurofilaments leading to axonal radial growth independent to myelin formation [175]. In addition, OGs are required for the clustering of sodium channels essential for the propagation of electrical impulses required for normal physiological functioning [176]. Furthermore, the development of axonal swelling and degeneration in the absence of the myelin protein PLP suggests axons require local OG support [177]. Therefore, OGs and the myelin they produce are important for the viability of myelinated axons.

NGF induces myelination in the CNS [154, 178] and PNS [86]. NGF regulates various cellular events including OG regeneration [155] and the protective and survival of OGs and OPs [78, 154, 155] **(Table 1)**. During the early stages of MS, mature OGs [52] and OPs attempt to repair immune mediated myelin damage [46-49]. However, this process is limited as OGs are not able to fully re-myelinate. In addition, successive attacks on the CNS results in less effective re-myelination by OGs. TNF α contributes to demyelination, OG damage, and OG apoptosis [179] through TNFR1 [76, 77]. However, TNF α -induced OG cell death is prevented by astrocyte conditioned medium [78] because astrocytes have been shown to synthesize NGF mRNA and protein [180, 181]. Furthermore, neutralizing antibodies against NGF inhibit the anti-apoptotic effects of astrocyte conditioned medium [78]. This study demonstrated that NGF protects OGs from TNF α -mediated apoptosis through the Akt pathway [78], which is required for OG survival [182]. TNF α can also promote OP proliferation and myelination through TNFR2 in the presence of NGF [78], although, this mechanism is unknown.

NGF has also been demonstrated to enhance OG process regeneration and proliferation [155]. Studies suggest that $p75^{NTR}$ receptor is involved in migratory activity of OPs [183, 184]. OPs expressing $p75^{NTR}$ have been identified in brain lesions of MS patients [185]. This suggests NGF may modulate OP migration along radial astroglia through the up-regulation of $p75^{NTR}$ [186]. It has been demonstrated that OP proliferate prior to re-myelinating denuded axons [187, 188]. It has also been demonstrated that “shadow” plaques contain thinly myelinated fibers, suggesting re-myelination of MS lesions [189]. Failure of myelin repair is therefore due to the loss of OGs and the failure of OPs to

migrate and proliferate sufficiently to affect re-myelination of the lesions [188]. This indicates NGF is a crucial factor in promoting migration, proliferation and differentiation of OPs required for re-myelination.

2.6.3. NGF STIMULATES RE-MYELINATION BY SCHWANN CELLS: IMPLICATIONS IN CNS RE-MYELINATION

Other myelinating cells such as Schwann cells of the PNS, are also thought to have the ability to re-myelinate axons in the CNS [190] (**Table 1**). The exact mechanism of spontaneous CNS myelination by Schwann cells remains unclear, but NGF has been shown to promote Schwann cell migration leading to re-myelinating activity in the CNS. It has been reported that following peripheral nerve injury, the release of IL-1 from macrophages stimulate NGF expression in PNS Schwann cells [191]. Similarly, Schwann cells located in the subpial region of the injured SC can be stimulated to produce NGF by activated microglia expressing IL-1 and other cytokines [191, 192]. Interestingly, Schwann cells have been shown to migrate and re-myelinate axons in MS lesions near peripheral nerve entry zones in the SC [193-195] . Schwann cell migration into demyelinated areas of the CNS is aided by glial bridges resulting from irregularities in astrocytes that cause the breakdown of the glia limitans which allows Schwann cells to penetrate the CNS [196]. Schwann cell migration is mediated by NGF via $p75^{NTR}$ [184]. NGF acts as a chemokinetic and chemo-attractant cue for Schwann cells [197]. These findings suggest that the increased expression of NGF in the CNS, is an important step for the migration of Schwann cells into the CNS to repair areas of myelin damage.

NGF is also a chemokinetic molecule for Schwann cell precursors [198], that may serve as a source of mature Schwann cells required for myelination at the injured site.

2.7. NGF REGULATION OF BDNF: IMPLICATIONS IN MYELINATION

BDNF has been shown to promote OP proliferation [199, 200], OG differentiation and affect OP development through the TrkB/MAPK pathway [69, 73, 201]. BDNF also has an integral role in myelin formation [72, 74] that is evident by its ability to increase the expression of the key structural proteins that comprise myelin which include: MBP [202], MAG, and PLP [73, 203]. Interestingly, NGF has also been shown to regulate the expression of MBP, PLP and MAG in OGs [201, 204], *via* interaction with $p75^{NTR}$ and TrkA receptors [201]. Analogous to NGF, evidence also supports the involvement of BDNF in re-myelination of MS induced SC lesions [200, 205]. Specifically, the delivery of exogenous BDNF to the CNS of mice induced to a state of EAE resulted in a significant increase in re-myelination at demyelinated areas [205]. Furthermore, BDNF knockout studies show that the absence of BDNF in EAE animals significantly increased the structural damage to myelin [206].

Based on the current literature NGF and BDNF are able to reciprocally regulate each other. For example, *in vitro* studies demonstrate that administration of exogenous NGF induces the gene expression of BDNF mRNA in DRG neurons [82] thereby supporting the concept that BDNF's actions can be mediated by NGF [67, 79, 80, 82-85]. However, the exact molecular mechanisms by which NGF governs BDNF expression have not been

fully established. *In vivo* EAE studies have also demonstrated the ability of NGF to induce BDNF expression [207]. Furthermore, research also suggests that BDNF can exert its biological effects via the TrkA receptor for which NGF normally signals [83, 208, 209]. Conversely, BDNF can induce NGF secretion from neurons [210]. These studies demonstrate the importance of the reciprocal synergistic relationship between NGF and BDNF and acknowledge their potential implications in myelin formation and repair.

2.8. REGULATION OF TNF α: IMPLICATIONS IN MYELINATION

TNF α is a cytokine that has been widely implicated in the underlying pathology of MS [211, 212]. For example, our recently published work has shown that TNF α expression is significantly up-regulated in the DRG and SC of MBP EAE rats at 12 days post-induction which correlates with peak neurological disability [36, 213]. Specifically, TNF α is thought to induce OG apoptosis and demyelination [179] via TNFR1 [76, 77]. Interestingly TNF α has also been shown to induce the production of NGF in response to inflammation [63]. However, recent evidence suggests that NGF is a key regulator of the biological activity of TNF α. For example, TNF α-induced OG apoptosis can be prevented with the delivery of NGF from astrocytes which inhibits OG damage and loss of mitochondrial potential [78]. NGF acts via $p75^{NTR}$ to preferentially induce TNF α signaling via TNFR2 to promote OP proliferation and myelination [66, 179, 214]. Based on NGF's ability to regulate TNF α activity, it is plausible that MS patients have lower baseline levels of NGF or reduced $p75^{NTR}$ that preferentially drives TNF α signaling through the TNFR1 pathogenic pathway. Thus, NGF plays a critical role in modulating

the inflammatory and/or cytotoxic effects of immune system derived TNF α. As a result, this underlies the significance of NGF's role promoting the survival and protection of OGs and OPs, which are critical components essential for myelin repair process.

2.9. NGF - BDNF - TNF α SIGNALING TRIAD: IMPLICATIONS IN MYELINATION

The interconnected signaling network between neurotrophins and cytokines demonstrates the importance of this critical signaling triad to the regulation of myelin repair **(Figure 1a & 1b)**. As such, we have proposed a model of NGF-BDNF-TNF **α** signaling that is consistent with autoimmune theory of MS. This model is consistent with our previous published research that demonstrated the importance of the immune system mediated induction of the DRG in an animal model of MS [88, 213, 215]. Based on our research, we have shown that the anatomical connection between the DRG and SC via the dorsal roots is an imperative connective pathway by which this triad signaling regulates myelination [213]. Specifically we have shown that the antigenic activation of Th1 cells in the blood, leads to their migration into the DRG where they continue to liberate the production of inflammatory cytokines such as TNF α. This event subsequently activates the enhanced production of BDNF from small to medium sized DRG sensory neurons which subsequently is transported to the SC via the dorsal roots [213]. Henceforth, this research also acknowledges the importance of targeting the DRG – SC interconnected pathway as an alternative approach that can be exploited to facilitate re-myelination of immune system mediated myelin damage.

2.10. CURRENT TREATMENTS FOR RELAPSING REMITTING MS

To date there are no FDA approved disease-modifying therapies (DMT) for primary progression MS (PPMS). In addition, there are only minimal treatment options for secondary progressive MS (SPMS). However, at present there are only 8 DMTs approved for relapse-remitting MS (RRMS): IFN-β products (Avonex®, Rebif®, Betaseron®, and Extavia®), glatiramer acetate (Copaxone®), mitoxantrone (Novantrone®), natalizunab (Tysabri®) and fingolimod (Gilenya®) [216]. Although the exact mechanism for IFN-β products is unknown, they generally function by reducing T cell infiltration into the CNS and regulate pro- and anti-inflammatory cytokines by shifting the balance from inflammatory Th1 to anti-inflammatory Th2 lymphocytes [217]. However, research has suggested that IFN-β is a potent inducer of NGF [218]. For example, pre-treatment of T cells with IFN-β *in vitro*, and treatment of T cells derived from MS patients with IFN-β (*in vivo)* triggers a significant increase in NGF mRNA and protein production by human brain microvascular cells [219]. This suggests that IFN-β may *indirectly* contribute to re-myelination *via* IFN-β –induced NGF production. Although, the precise mechanism of action of glatiramer acetate (GA) is unknown, it is thought to exert its beneficial effects by suppressing T cell activation, and possessing neuroprotective properties through the induced production of BDNF [220]. As a result, the resultant GA-induced BDNF production may indirectly promote NGF expression that facilitates myelin repair. Mitoxantrone intercalates into DNA and interferes with DNA repair by effectively inhibiting topoisomerase II, and causing cross-linking and strand breaks resulting in immunosuppression, inhibition of monocyte and lymphocyte migration, and decreased

secretion TNF α, IL-2, and IFNγ [216, 221]. However, its marginal efficacy, compounded with cardiac side effects have made it a less favorable treatment option for RRMS [222]. Natalizumab acts by binding to CD49 on the very late antigen-4 (VLA-4) receptor, thereby, preventing immune cell adhesion to the BBB and invasion of the CNS of leukocytes [223]. In this manner, it prevents the migration of activated Th1 cells across the BBB. Fingolimod is a new oral MS treatment for RRMS that entraps activated Th1 cells in the lymph nodes, thereby preventing the egress of activated Th1 cells from the lymph nodes into the peripheral blood [224]. Henceforth, despite all the currently available MS treatments, these drugs can only slow disease progression and reduce the frequency and intensity of MS attacks. At present, there is no cure for MS since none of the treatments can ***directly*** re-myelinate areas affected by immune system mediated myelin damage.

2.11. NGF: A PROMISING TREATMENT OPTION FOR MS

Numerous EAE studies conducted in a various types of EAE animal models consistently support the beneficial role of NGF in regulating re-myelination and the structural integrity of myelin [89, 201, 225-229]. In addition, human studies involving RRMS patients support the beneficial effects of NGF in specific regard to improving cognitive performance [230]. Furthermore, other human studies report that RRMS patients secrete lower levels of NGF and BDNF compared to healthy controls which suggests that the sub-optimal levels of neurotrophins predispose MS patients to lesion formation [231]. As a result, NGF is recognized as a promising treatment option for MS that has direct effects

on repairing immune system mediated myelin damage. However, the development of NGF-based therapies has been challenging due to issues surrounding its delivery and safety. For example, following the systemic administration of NGF, it fails to cross the BBB and therefore is unable to exert its beneficial effects on myelin within the CNS [232, 233]. Henceforth, significant pharmaceutical manipulation of the drug formulation is yet to be achieved to ensure that NGF can reach its intended target within the CNS. For example, studies involving Alzheimer's disease have attempted to circumvent the BBB by directly administering an intra-cerebroventricular infusion of NGF into the brain ventricles. However, this led to the development of severe adverse effects that compromised drug efficacy [234, 235]. Due to the challenges associated with the systemic and intra-cerebroventricular delivery of NGF other alternative formulation strategies have been developed to deliver NGF directly into the CNS. For example, e*x vivo* gene delivery of autologous fibroblasts genetically modified to produce and secrete NGF has been shown to be a promising option that can enhance drug delivery to the target while minimizing adverse effects associated with its treatment [236, 237]. The favorable pre-clinical results using NGF gene therapy has also showed promising effects in human clinical trials for Alzheimer's disease [238]. A phase 1 clinical trial of genetically engineered autologous fibroblasts genetically engineered to secrete human NGF improved cognitive decline with the absence of long- term adverse effects in most patients [239]. A second phase 1 randomized study to assess the safety and tolerability a human NGF genetically engineered into an adeno-associated virus vector (CERE-110) is currently ongoing [240, 241]. Encapsulated cell biodelivery represents a promising alternative that is both implantable and retrievable [242]. A long-term (12 months) phase

1 clinical trial studying the tolerability and safety of implants of an encapsulated cell biodelivery device housing human NGF-secreting cell line in patients with Alzheimer's, demonstrated the implantation and removal of the device is safe, well tolerated, and in some patients, associated with positive neurological outcome [243, 244]. Other NGF-based formulation strategies includes small molecule mimetics that possess intrinsic NGF activity and enhanced pharmacokinetic profiles [232]. These peptide mimetics may be less immunogenic, possess greater stability against protein degradation and have a greater ability to permeate the BBB [245]. NGF-mimetic peptides include L1L4, which has have demonstrated good NGF-like activity *in vitro* by inducing differentiation of the DRG, TrkA phosphorylation, and differentiation of PC12 cells [246]. L1L4 has been shown to be effective in reducing neuropathic pain [246]. Non-invasive routes of NGF-based therapies are also being considered including ocular and intranasal delivery. Recombinant NGF, hNGF-61, administration intra-nasally proved to be an effective means of NGF delivery into the CNS in therapeutic doses without unwanted side effects [247].

2.12. CONCLUSION

Currently available treatment strategies for MS are primarily targeted towards modulating or suppressing the immune system. The possibility of preventing or reversing the progression of neurological disability associated with MS depends on the ability of novel therapeutic strategies to promote or enhance re-myelination. Despite the significant treatment advances made in the field of MS, the discovery of a novel treatment that re-

myelinate damaged axons at the site of injury has been challenging. Recent studies support further investigation into NGF as a promising new agent that can be added to the armamentarium of potential pipeline treatments for MS. Unveiling the molecular signaling between neurotrophins and cytokines [36, 88, 213] and their upstream transcriptional regulators [248, 249] may lead to the discovery of other novel therapeutic targets that can be exploited for the purposes of facilitating myelin repair. Based on the critical molecular signaling triad between TNF α, NGF and BDNF, the initial induction of TNF α during an MS attack normally would induce the production of BDNF and NGF to prevent myelin damage from occurring. However, MS patients may be predisposed to develop MS lesions due to their inherent inability to produce sufficient quantities of NGF and BDNF that could have attenuated the initial immune mediated pathology triggered by TNF α. Despite the plethora of evidence to support the beneficial role of NGF in myelin repair, the ongoing challenges of drug delivery and adverse reactions associated with its treatment still prevail.

Studies on neurodegenerative diseases, such as Alzheimer's disease, demonstrate NGF can successfully recover cognitive decline in human patients. Moreover, these studies demonstrate NGF can be administered safely and with minimal adverse effects associated with gene therapy methods. While, clinical trials are ongoing for NGF-based treatment in Alzheimer's disease, studies on NGF-based therapeutics in MS is far more limited. Overall there is significant evidence to support the role of NGF in myelin repair, which defines a new therapeutic strategy for MS. Thus, significant potential exists in regard to the use of NGF-based therapeutics. The advancements made in regard to NGF

therapeutics will have the diverse applicability to other white matter disorders and may lead to novel interventions for medical conditions such as spinal cord injury.

2.13. TABLES

Table 1. NGF Biological Effects on Mediating Myelination	
Target	**Biological Activity**
OGs	Promotes protection, survival, and proliferation [78, 154, 155]
OPs	Promotes protection, survival, and migration [152, 186]
Schwann cells	Promotes migration, serves as chemo-attractant and chemo-kinetic cue [184, 197]
Schwann cell precursor	Serves as a chemo-kinetic cue [198]
Neurons and axons	Promotes neuronal survival, axonal regeneration, branching, and elongation [85, 153, 173, 174]
CNS Myelin	Increases the expression of myelin proteins [201, 204]

2.14. FIGURES

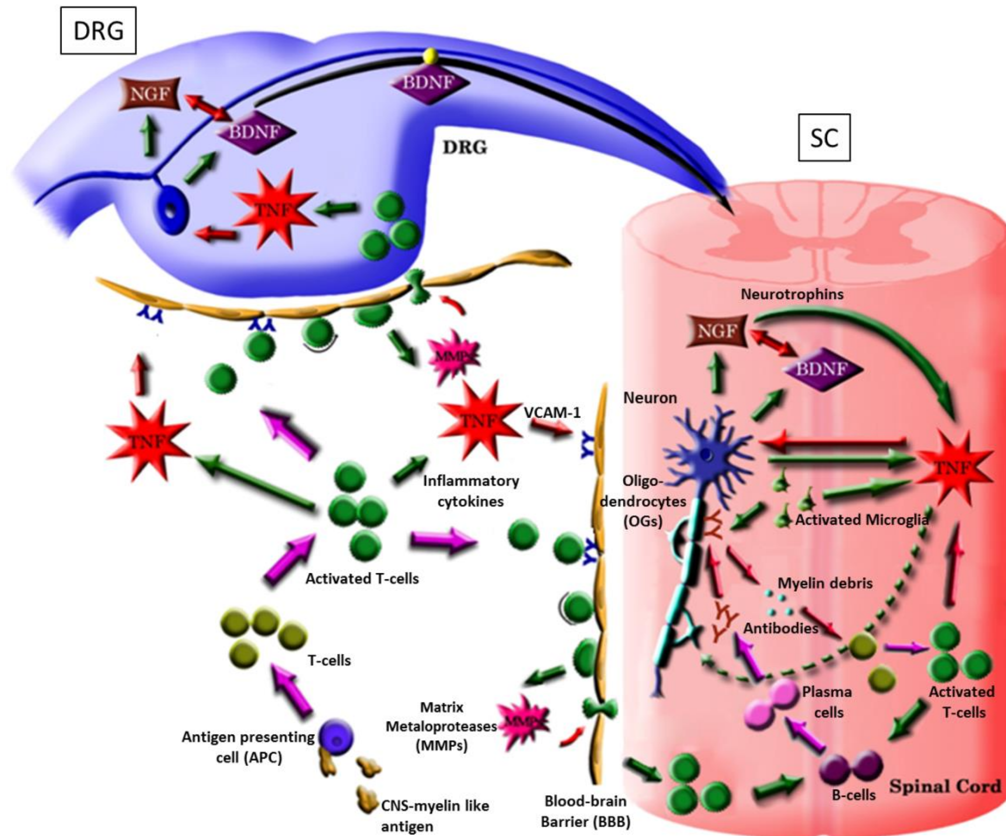

Figure 1a: Autoimmune model of MS-myelin damage. Exposure to an antigenic protein that shares a similar homology to proteins that compose CNS myelin, triggers the activation of inflammatory Th1 cells in the blood. Once activated, the Th1 cells release inflammatory cytokines (TNF α, IL-12, IFNγ) which enhance the proliferation and lineage expansion of the specific Th1 cells that were activated by the initial antigenic protein that triggered the immune response. As the immune response progresses, Th1 cells eventually migrate into DRG via the high permeable vascular capillaries where they continue to produce more inflammatory cytokines. The production of Th1 derived inflammatory cytokines within the DRG, prime the activation of sensory neurons to

recruit their involvement in the production of cytokines such as TNF α. The TNF α collectively derived from Th1 lymphocytes and sensory neurons subsequently activates other sensory neurons to produce neurotrophins such as BDNF and NGF. As a result, Th1 cells, cytokines, and neurotrophins [213] subsequently undergo anterograde transport to the SC via the dorsal roots. Inside the SC, Th1 lymphocytes become re-activated in presence of local CNS myelin proteins that share the same or similar amino-acid sequence of the initial antigenic protein that initiated the immune response in the peripheral blood. These re-activated Th1 cells continue to produce TNF α, and other inflammatory cytokines which once again, facilitate the lineage expansion of these Th1 cells in the CNS. The subsequent increase in central TNF α production from reactivated Th1 lymphocytes again initiates the induction of central derived BDNF and NGF which are critical in the regulation of myelin repair.

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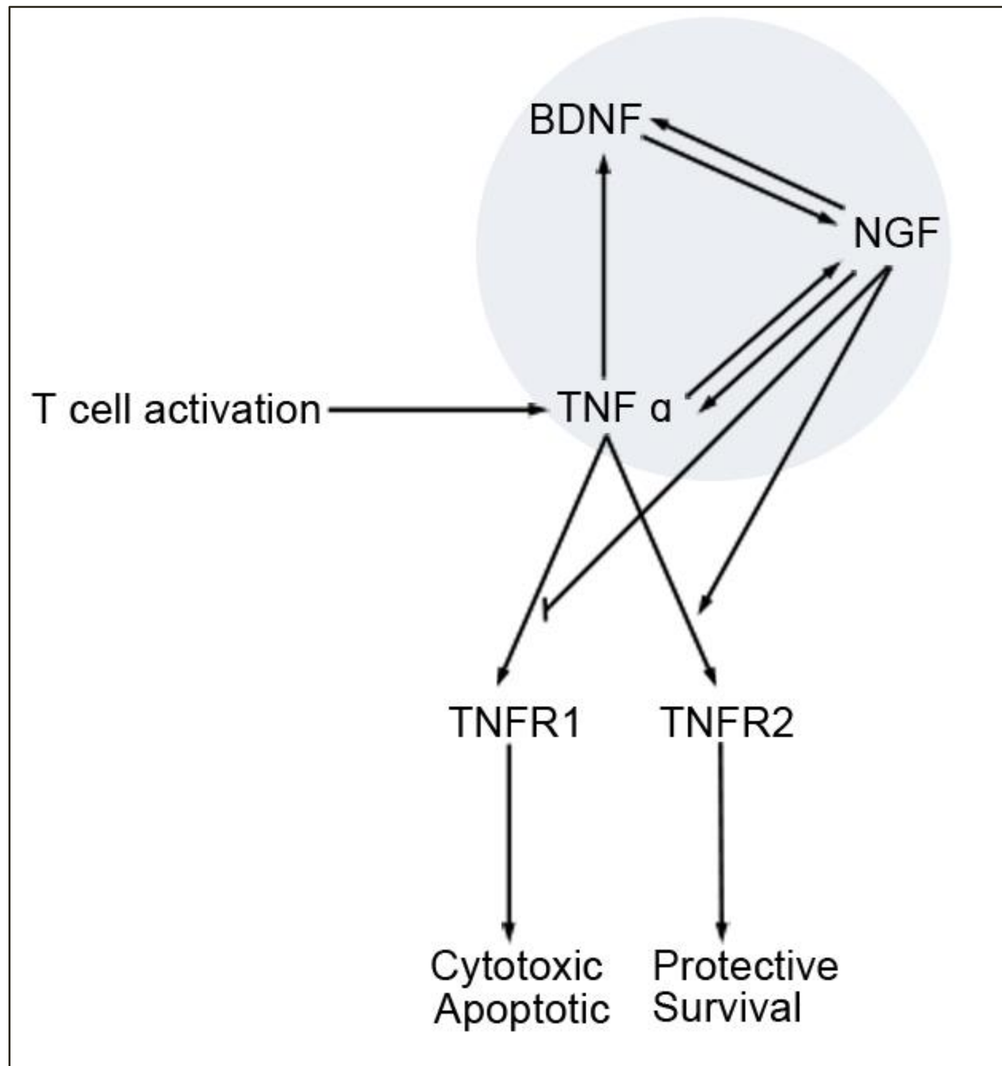

Figure 1b: A schematic representation of the NGF, BDNF and TNF α signaling triad. T cell activation results in TNF α expression leading to the induction BDNF and NGF expression. BDNF can induce NGF secretion and NGF further induces BDNF expression. NGF effects TNF α signaling pathways by suppressing TNFR1-mediated apoptosis and cell damage and promoting preferential signaling through TNFR2 which leads to protection and survival.

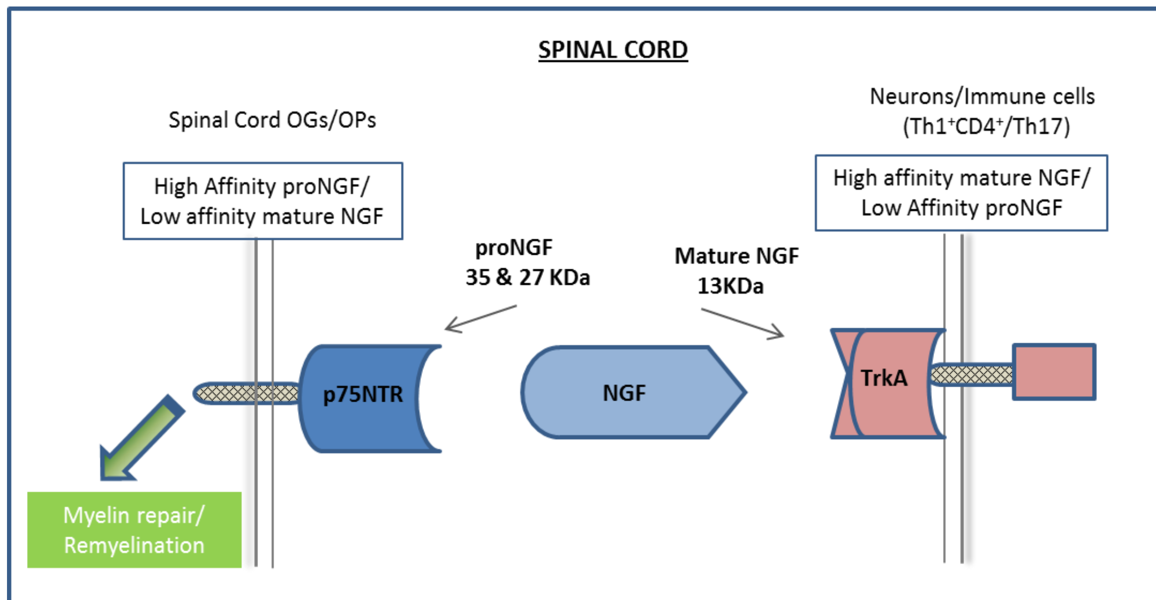

Figure 2: proNGF (35 & 27 kDa forms) and mature NGF (13 kDa) preferentially interact with p75^{NTR} and TrkA receptors, respectively, to produce a differential cellular response in the SC. The mature 13kDa form of NGF preferentially signals *via* the TrkA [127-129]. However, proNGF preferentially interacts with high-affinity at the p75^{NTR} and mediates biological actions distinct from those of TrkA receptor [130, 139]. Published studies show that OGs express p75^{NTR} [154, 250] as well as TrkA [251], suggesting that proNGF or mature forms of NGF can promote myelination *via* p75^{NTR} and/or TrkA mediated activity.

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PROLOGUE TO CHAPTER 3: RATIONALE FOR MANUSCRIPT

One of the hallmark symptoms commonly encountered by MS patients is neuropathic pain (NPP) [1-3]. NPP is a chronic pain syndrome whose pathology has been linked to the abnormal synchronous hyper-excitability of the dorsal horn neurons of the SC [4]. Patients with NPP suffer sensory abnormalities such as numbness, burning, shooting pain with the feeling of pins and needles, which vary in severity intensity and location [5, 6]. NPP is caused by drug, injury or disease-induced damage to the peripheral or central nerve fibers involved in the synaptic transmission of pain [4, 7, 8]. MS is a disease that produces targeted damage to both peripheral and central nerve fibers. As such, NPP is the second worst disease induced symptom suffered by patients with MS [1]. Interestingly, several of the pathological mediators that drive the underlying pathogenesis of NPP also drive the pathogenesis of MS [9-11]. Studies suggest that fractalkine (CX3CL1) and its receptor (CX3CR1) may be involved in the induction of NPP [12-15]. As a result, we set out to investigate the gene and protein expression changes of CX3CL1 and CX3CR1 in the DRG and SC using an animal model of MS. By examining the time dependent changes of CX3CL1 and CX3CR1 during the early, acute, inflammatory stage of EAE we can gain a better understanding of the factors involved in MS associated pain induction and provide insights to early indicators of the disease process.

This study is of particular significance to my thesis because it supports the role of DRG activation in the induction of MS, which is a key conceptual aspect behind the rationale for my project. Further, my involvement in this study allowed me to develop proficiency

in protein analysis *via* immunohistochemistry (IHC) that was essential to my project. I was also able to develop an understanding of statistical analyses that were pertinent in the execution and completion of my own project. Furthermore, this study allowed me to familiarize myself with other techniques important for my own study including protein analyses techniques, such as western blot (WB) and enzyme linked immunosorbent assay (ELISA), as well as, gene analysis *via* quantitative real time polymerase chain reaction (qRT-PCR).

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CHAPTER 3: ELEVATED EXPRESSION OF FRACTALKINE (CX3CL1) AND FRACTALKINE RECEPTOR (CX3CR1) IN THE DORSAL ROOT GANGLIA (DRG) AND SPINAL CORD (SC) IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE): IMPLICATIONS IN MULTIPLE SCLEROSIS (MS) – INDUCED NEUROPATHIC PAIN (NPP)

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STATEMENT OF CONTRIBUTION

For this manuscript published October 2013, I was responsible for performing IHC and imaging, specifically for co-labeled CX3CR1 and CD68 in the SC In addition, I assisted in the statistical analysis of ELISA and qRt-PCR data.

CHAPTER 3

3.1 ABSTRACT

Multiple sclerosis (MS) is a central nervous system (CNS) disease resulting from a targeted autoimmune-mediated attack on myelin proteins in the CNS. The release of Th1 inflammatory mediators in the CNS activates macrophages, antibodies, and microglia resulting in myelin damage and the induction of neuropathic pain (NPP). Molecular signaling through fractalkine (CX3CL1), a nociceptive chemokine, via its receptor (CX3CR1) is thought to be associated with MS-induced NPP. An experimental autoimmune encephalomyelitis (EAE) model of MS was utilized to assess time dependent gene and protein expression changes of CX3CL1 and CX3CR1 in the dorsal root ganglia (DRG) and spinal cord (SC) 12 days after EAE induction compared to controls. This increased expression correlated with behavioural thermal sensory abnormalities consistent with NPP. Furthermore, this increased expression correlated with the peak neurological disability caused by EAE induction. This is the first study to identify CX3CL1 signaling through CX3CR1 via the DRG/SC anatomical connection that represents a critical pathway involved in NPP induction in an EAE model of MS.

Keywords: CX3CL1, CX3CR1, experimental autoimmune encephalomyelitis, multiple sclerosis, neuropathic pain, dorsal root ganglia, spinal cord.

3.2. INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) which is characterized by inflammation and subsequent demyelination of brain and spinal cord (SC) [1, 2]. Although the exact pathophysiology of MS is still unknown, it is associated with CNS infiltration of activated inflammatory Th1 cells resulting in axonal myelin damage and subsequent neuronal destruction. The targeted immune mediated destruction of CNS myelin results in a variety of neurological deficits that include but are not limited to ataxia, cognitive dysfunction, weakness, fatigue, motor deficits and sensory abnormalities such as neuropathic pain (NPP) [1, 3-5].

Chemokines are a family of small cytokines that function as key mediators which control the response of leukocytes in areas of inflammation. They also act as chemotactic cues for leukocytes via interactions with their G-protein coupled, cell membrane-spanning receptors. Currently, fifty chemokines have been identified, which have been divided into four subgroups of chemokines: XC, CC, CXC and CX3C [6]. Synthesis of chemokines occurs rapidly within infected or damaged tissues. They are thought to drive chronic neuroinflammatory processes in order to attract appropriate cell populations to combat invading organisms and repair damaged CNS tissues [6]. Recent studies aimed at using chemokine antagonists support the importance of chemokines in pain induction, as blocking their molecular signaling has been suggested to ameliorate neurological deficits such as NPP in inflammatory autoimmune disorders, such as MS [7, 8].

CX3CL1 (fractalkine) is the only member of the fourth class of chemokines, with a CX3C motif in the mucine-like domain [9, 10]. It is unique in that it is tethered to a cell membrane and is cleaved after an excitotoxic stimulus, to produce a soluble, diffusible protein [11]. CX3CL1 is usually expressed in the normal rodent CNS tissue by different neuronal cell subtypes [12]. In addition, it is also expressed in monocytes, natural killer (NK) cells and smooth muscle cells [13]. Recent evidence has shown that CX3CL1 and its receptor CX3CR1 are known to be involved in the pathogenesis of other clinical diseases such as rheumatoid arthritis, chronic pancreatitis and NPP [14-17] through their ability to regulate neuronal-microglial communication [18]. In the CNS, CX3CL1 is highly expressed by neurons while CX3CR1 is only expressed by microglia [12, 19]. Specifically, studies have shown that SC microglia expression of CX3CR1 significantly increases in animal models of NPP relative to normal baseline levels of naive controls [13, 20, 21].

Several studies show that induction of NPP results in the synthesis and release of CX3CL1 in the sensory neurons of the dorsal root ganglion (DRG) [21, 22]. Furthermore, this increase is accompanied by the up-regulation of CX3CR1 in the SC microglia which correlates with the onset of NPP [21]. The most likely source of CX3CR1 in the SC of animals with NPP is resident microglia which is known to up-regulate CX3CR1 in response to injury [13, 21]. However, activated Th1-cells, transmigrating across the blood brain barrier may also be an additional source of increased CX3CR1-immunoreactivity detected in the SC during NPP [23].

Further evidence in support of the nociceptive role for CX3CL1 in NPP development comes from a study using intrathecal injections of CX3CL1 [22]. The results of this study showed that acute intrathecal injection of CX3CL1 resulted in the development of thermal hyperalgesia and mechanical allodynia in adult rats [24], while the administration of neutralizing antibodies against CX3CR1 attenuated the allodynia and hyperalgesia. Taken together, these results directly link the molecular signaling of CX3CL1 through CX3CR1 to the induction of pain [24, 25]. However, in a spared nerve injury model performed in CX3CR1 knockout mice, researchers showed increased allodynia thereby suggesting an alternative nociceptive signaling pathway for CX3CL1 besides that which was soley elicited through CX3CR1[22].

We *hypothesize* that the molecular signaling of CX3CL1 through its receptor CX3CR1 via the anatomical DRG/SC connection, represents a critical pathway involved in the induction of MS–induced NPP.

In order to confirm the role of CX3CL1 and CX3CR1 in MS-induced NPP, we assessed the gene and protein expression of CX3CL1 and CX3CR1 in a rat EAE model induced by myelin basic protein (MBP) [26]. Our study shows significant increases in the DRG and SC of both CX3CL1 and its receptor CX3CR1. Our results also confirmed that the increased CX3CL1 and CX3CR1 levels correlate with progression of behavioral sensory abnormalities which is consistent with that of MS–induced NPP. Moreover, we also show a detailed immunohistochemical (IHC) analysis of the cellular distribution of CX3CL1 and CX3CR1 in the EAE SC to identify the cellular sources that contribute to the

expression of CX3CL1/CX3CR1. This study confirms the involvement of CX3CL1 signaling through CX3CR1 in the induction of in MS-induced NPP.

3.3. MATERIALS AND METHODS

3.3.1. EAE INDUCTION

Adolescent female Lewis rats 6-8 weeks of age, weighing 135-150 g (Charles River, Montreal, QC) were induced to a state of EAE using MBP in accordance with in-house methods previously published [26, 27]. Briefly, rats were maintained at 22°C in a room with automatic light/dark cycles of 12/12 hours. The rats were randomly assigned to three experimental groups: naïve control (NC), active control (AC), and EAE. There were 5 pre-determined time points for sacrifice identified at 3, 6, 9, 12 and 15 days post inoculation (DPI) in the AC and EAE groups. For example, EAE animals euthanized at day 3 would be referred to as EAE3 versus AC control animals euthanized at day 3 would be referred to as AC3. All animal experiments in the present study were conducted according to protocols approved (#10-024/1/2) by the University of Manitoba Animal Protocol Management and Review Committee, in full compliance with the Canadian Council on Animal Care. Neurological disability is scored according to the criteria outlined in **Table 1**.

3.3.2. TISSUE HARVESTING FOR CRYOSECTIONING

For IHC analysis of protein expression, animals were perfusion fixed with 4% paraformaldehyde as previously described [26, 27]. Spinal columns were dissected and decalcified for subsequent cryostat sectioning (10 µm sections) according to previously described and published in-house protocols [28]. The tissue was collected at the various time-dependent stages of disease progression. SC and DRG were collected at days 3, 6, 9, 12 and 15 after inoculations.

3.3.3. GENE/PROTEIN ANALYSIS

DRG and SC tissues were harvested for gene and protein expression analysis of CX3CL1 and its receptor CX3CR1, as previously described [26, 27]. Freshly harvested tissue was extracted and stored in RNA later stabilization reagent (Qiagen, cat. no. 76106, Washington DC, USA) until processed. Total RNA and protein were extracted using commercially available kits (All Prep; Qiagen) as described previously [26, 27].

3.3.4. THERMAL SENSORY TESTING

Withdrawal latencies to a radiant heat stimulus [29] were assessed for each rat using a Model 336G Plantar/Tail Stimulator Analgesia Meter (IITC Life Sciences, Woodland Hills, CA) according to previously published in-house methods [5]. The time required to move the paw or tail from the heat source was recorded as the withdrawal latency. Rats

were habituated to the testing apparatus for 30 minutes, 2 days prior to any testing and for 10 minutes prior to testing on each test day. A standardized 60% light output intensity setting was used for thermal testing. Each experimental group was tested every day after inoculation. Region specific withdrawal responses consisted of licking the paws and flicking the tail in response to the heat stimulus. Withdrawal latencies were recorded in seconds with a maximum of 20 second cut-off point programmed into the timer to prevent tissue damage. Based on our previous published methodology, withdrawal latency was recorded three separate times (seconds) for each paw and tail and average withdrawal latency was calculated. These latencies were then normalized to baseline values and presented as percentages [5, 26].

3.3.5. MECHANICAL ALLODYNIA

To quantify mechanical allodynia, rats were placed in lucite cubicles over top of a metal mesh floor and mechanical stimuli applied to each hind paw with a 1.0 mm von Frey filament attached to a digitized strain gauge [30]. The maximum force generated before withdrawal is recorded for each hind paw over three trials and averaged.

3.3.6. IHC STAINING

IHC was conducted on 10µm cryostat sections to detect cellular location of the protein expression according to previously published in-house methods [26, 27]. Double-labeled IHC analysis was conducted using polyclonal antibodies against the neuronal marker

NeuN (1:100; Chemicon, Billerica, MA, USA), astrocyte marker glial fibrillary acidic protein (GFAP) (1:100; Santa Cruz, CA, USA), microglia marker CD68 (ED1) (1:100, Santa Cruz, CA, USA) were conducted in conjunction with the polyclonal antibody for CX3CL1 (1:100; eBioscience, San Diego, CA, USA) and CX3CR1 (1:100; eBioscience, San Diego, CA, USA). Secondary antibodies were goat anti-mouse FITC (1:100, Jackson, West Grove, PA, USA) and goat anti-rabbit TRITC (1:100; Jackson, West Grove, PA, USA). The slides were imaged using the Nikon DS-US camera and images were captured at the same exposure times and colorized in Image pro plus 6.2. Image sizing, black background balancing and final collation for publication were performed using Adobe Creative Suite 2 v9.0.2 (Adobe Systems Inc., San Jose, CA, USA). No image manipulations were performed other than those described.

3.3.7. REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (REAL TIME RT-PCR)

Real time RT-PCR was conducted on DRG and SC as per previously published in-house methods [26, 27]. The PCR reaction was performed using a Light-Cycler-DNA master SYBR green 1 kit following manufacturers protocols (Bio-Rad, Hercules, CA, USA). CX3CL1 primers were: *forward:* 5'-gaattcctggcgggtcagcacctcggcata-3'; *reverse*: 5'-aagcttttacagggcagcggtctggtggt-3' at an annealing temperature of 60°C. CX3CR1 primers were: forward: 5'-agctgctcaggacctcaccat-3'; reverse; 5,-gttgtggaggccctcatggctgat-3' at an annealing temperature of 60°C. The cDNAs were amplified by 35 cycles of PCR. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase

(GAPDH). GAPDH is an enzyme associated with cell metabolism and is used as a standard housekeeping gene for expression pattern comparisons [31, 32]. GAPDH primers were commercially available from SuperArray. The quantification technique used the standard curve method.

3.3.8. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Total protein was extracted from the SC and DRG as described above and total protein concentration assessed using the Bradford assay [33]. The protein concentrations of the samples were adjusted to 10 μg total protein for CX3CL1and 1 μg total protein for CX3CR1 in the sample volume of 100 μl. Sandwich-style ELISA was performed using the RayBio rat CX3CL1 ELISA kit (RayBio, Norcross, GA, USA) and rat chemokine CX3CR1 ELISA kit (Mybiosource, San Diego, CA ,USA) according to the manufacturer's instructions. CX3CL1 and CX3CR1 contents were measured from standard curve runs for each plate (linear range of 0–2000 ng for CX3CL1; 0-10 ng for CX3CR1). Samples from the groups of AC and EAE and the NC rats were determined in each run. Each sample was assayed with 6 replicates per ELISA.

3.3.9. STATISTICAL ANALYSIS

Statistics was performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical analysis for ELISA and Real Time-PCR (RT-PCR) was performed using ANOVA with Tukey's Multiple

Comparison post hoc test. For the behavioral analysis, Student's *t*-test was used to confirm the significance of differences between the means of groups.

3.4. RESULTS

3.4.1. NEUROLOGICAL DISABILITY SCORES (NDS)

All animals in the EAE groups were scored for neurological disability according to a previously published in-house global neurological disability assessment tool [26, 27]. Prior to EAE6, none of the animals displayed clinical neurological deficits thereby scoring zero (Figure 1). At EAE6 neurological deficits began to be displayed in some animals in the form of tail weakness. By EAE9 *all animals* started to display clinical signs of neurological disability [0.57 ± 0.45; mean ± standard error of the mean (SEM)]. As a result, EAE9 was designated as the *"day of onset of neurological disability"*. Neurological disability progressively worsened upon daily assessment until EAE12 (peak disability; 6.42 ± 5.35), then subsided by EAE15 (1.5 ± 1.41) as the animals entered the remission/recovery phase of the disease [34] **(Figure 1).** The control groups (NC and AC) did not show any clinical signs of disability *(data not shown)*. The significant variation in presentation of NDS identified in this animal model of MS represents the characteristic variation of neurological deficits presented in humans with MS [1, 35].

3.4.2. ASSESSMENT OF THERMAL SENSORY TESTING – THERMAL HYPALGESIA

Sensitivity to noxious heat was measured in five specific anatomical domains which included the tail, right and left hind paws, right and left forepaws. Normalized thermal *tail* withdrawal latencies in EAE animals before and after disease onset are shown in **Figure 2(a)**. Thermal withdrawal latency was measured every day throughout the entire 14-day assessment period. Thermal withdrawal latencies were not measured on EAE15 as that was the day designated for animal sacrifice. The thermal sensory data indicated in **Figure 2(a)** identifies *day 0 (onset of neurological disability at EAE9)* as the first day in which all animals started to display clinical neurological deficits. All values were normalized to average baseline withdrawal latencies identified on the "x axis" as days -1 to -4 inclusive for comparative analysis at day 0 (onset of neurological disability) and days 1, 2, 3, 4 & 5 post-onset of neurological disability which were displayed as means ± SEM. A significantly elevated tail withdrawal latency was identified at day 0 compared to the average baseline withdrawal latency obtained from the withdrawal latencies recorded for the 4 days (-4 to -1) prior to the onset of neurological disability (day 0) (*$P < 0.023$; using a one sample t-test). These findings were consistent with that of thermal hypoalgesia. After this peak at day 0, tail withdrawal latencies decreased over days 2 to 5 where they remained stable and were not statistically significantly different from that of the average baseline withdrawal latency (days -1 to -4 inclusive). A total of n = 9 animals were used for comparative analysis at each of the predetermined experimental time points.

Similarly, a statistically significant increase (*$P < 0.05$) of the withdrawal latency characteristic of thermal hypoalgesia was observed in the *left hind limb* at day 4 (post-disease onset) compared to that of average baseline withdrawal latency (days -1 to -4 inclusive) and to day 0 (onset of neurological disability) as depicted in **Figure 2(b).** Furthermore, a statistically significant increase (*$P < 0.05$) of the withdrawal latency characteristic of thermal hypoalgesia was also observed in the *right and left forelimbs* at day 4 (post-disease onset) compared to that of average baseline withdrawal latency (days -1 to -4 inclusive), day 0 (onset of neurological disability) and days 1 and 2 after onset of neurological disability as depicted in **Figure 2(c)**. Our results are consistent with the results published by others that have also shown hypoalgesia prior to hyperalgesia in an EAE model of MS [36]. Due to the short duration of our EAE study (15 days) we were only able to demonstrate the early hypoalgesia component of MS induced NPP using our inflammatory MBP-induced EAE model of MS.

For mechanical allodynia, data were recorded from both hind limbs. An average normalized baseline response was obtained from days -1 to -4 (prior to the onset of neurological disability). Comparative analysis was conducted between average baseline values and those values obtained at day 0 and days 1, 2, 3, 4 and 5 after disease onset. No significant effects for mechanical allodynia were shown using a one sample t-test (*Data not shown*).

3.4.3. CX3CL1 GENE EXPRESSION ANALYSIS IN THE DRG

Real Time RT-PCR analysis was conducted on DRG isolated from the three experimental groups (EAE, NC and ACs), at the pre-determined experimental time points [**Figure 3(a)**]. The CX3CL1 mRNA expression was assessed in parallel with that of the housekeeping gene (GAPDH). NC animals (white bars) show CX3CL1 mRNA expression at 0.0419 ± 0.0058. AC animals (grey bars) shown a similar mRNA expression of CX3CL1 to NCs at all-time points (AC3 = 0.0456 ± 0.0028; AC6 = 0.0434 ± 0.0041; AC9 = 0.0449 ± 0.0047; AC12 = 0.0416 ± 0.0065; AC15 = 0.0540 ± 0.0035). In comparison, EAE animals (black bars) show a significant increase of CX3CL1 expression in DRG over NC at days 9, 12, and 15 post-inoculation (EAE9 = 0.0983 ± 0.0065, $P < 0.005$; EAE12 = 0.1323 ± 0.0154, $P < 0.005$ and EAE15 = 0.1208 ± 0.0102, $P < 0.005$). Furthermore, EAE animals show significant increase in mRNA expression over AC group at days 9, 12 and 15 ($P < 0.005$, $P < 0.005$ and $P < 0.005$). However, there is no significant change of CX3CL1 expression between EAE and NC at days 3 and 6 (EAE3 = 0.0520 ± 0.0006 and EAE6 = 0.0575 ± 0.0045). (ANOVA followed by Tukey's posthoc test) **[Figure 3(a)].**

3.4.4. CX3CL1 PROTEIN EXPRESSION ANALYSIS IN THE DRG BY ELISA

Total CX3CL1 protein expression is significantly altered in the lumbar dorsal root ganglia of EAE rats starting at day 12 after induction **[Figure 3(b)]** which directly correlated with the peak of neurological disability scores **(Figure 1)**. Results are given as ng CX3CL1 per 10 µg total protein for each sample. NC animals (white bars) show

CX3CL1 protein expression at 44.42 ± 13.58 ng/10 µg of total protein. AC animals (grey bars) show a similar protein expression of CX3CL1 to NC at all time points (AC3 = 43.85 ± 5.54 ng/10 µg total protein; AC6 = 51.22 ± 17.60 ng/10 µg total protein; AC9 = 41.86 ± 5.43 ng/10 µg total protein; AC12 = 41.25 ± 6.31 ng/10 µg total protein; AC15 = 30.87 ± 7.77 ng/10 µg total protein). In comparison, EAE animals (black bars) show a significant increase of CX3CL1 expression in DRG over AC at days 12 and 15 (EAE12 = 61.74 ± 10.98 ng/10 µg total protein, $P < .05$ and EAE15 = 53.48 ± 8.87 ng/10 µg total protein, $P < 0.05$). However, there is no significant change of CX3CL1 expression between EAE and AC at days 3, 6 and 9 (EAE3 = 48.18 ± 13.79 ng/10 µg total protein; EAE6 = 43.44 ± 4.33 ng/10 µg total protein and EAE9 = 56.82 ± 8.87 ng/10 µg total protein). (ANOVA followed by Tukey's posthoc test).

3.4.5. CX3CR1 GENE EXPRESSION ANALYSIS IN THE DRG

Total CX3CR1 mRNA expression in the DRG **[Figure 3(c)]** is significantly elevated at days 9 and 12 following EAE induction, which directly correlates with the onset and peak of neurological disability scoring **(Figure 1)**. Results are shown as a ratio of CX3CR1 mRNA to the housekeeping gene GAPDH. NC animals (white bars) show CX3CR1 mRNA expression at 0.0189 ± 0.0019. AC animals (grey bars) show a similar mRNA expression of CX3CR1 to NC at days 3, 6, 9 and 12 (AC3 = 0.0208 ± 0.0023; AC6 = 0.0254 ± 0.0009; AC9 = 0.0249 ± 0.0060; AC12 = 0.0287 ± 0.0073) except for the significant change at day 15 (AC15 = 0.0349 ± 0.0032, $P < 0.01$). In comparison, EAE animals (black bars) show a significant increase in CX3CR1 expression in DRG over NC

at days 9 and 12 (EAE9 = 0.0379 ± 0.0028, $P < 0.005$ and EAE12 = 0.0327 ± 0.0252, $P < 0.01$). However, there is no significant change of CX3CR1 expression between EAE and NC at days 3, 6 and 15 (EAE3 = 0.0228 ± 0.0026, EAE6 = 0.0248 ± 0.0041 and EAE15 = 0.0228 ± 0.0022). However, EAE animals did show a significant increase in mRNA expression over AC group at days 9 and 15 ($P < 0.05$ and $P < 0.05$ respectively) shown in **Figure 3(c)**. (ANOVA followed by Tukey's posthoc test).

3.4.6. CX3CR1 PROTEIN EXPRESSION ANALYSIS IN THE DRG BY ELISA

Total CX3CR1 protein expression in the DRG is also altered at day 12 following EAE induction which corresponds to the peak neurological disability scores **[Figure 3(d)]**. Results are given as ng CX3CR1 per 1 µg total protein for each sample. NC (white bars) animals show a baseline level of CX3CR1 in the DRG of 2.24 ± 0.24 ng/1 µg total protein. AC animals (grey bars) show a similar expression level of CX3CR1 compared to that of NC animals at all time points assessed (AC3 = 2.08 ± 0.15 ng/1 µg total protein; AC6 = 2.90 ± 0.85 ng/1 µg total protein; AC9 = 2.21 ± 0.22 ng/1 µg total protein; AC12 = 2.15 ± 0.13 ng/1 µg total protein and AC15 = 2.34 ± 0.29 ng/1 µg total protein). In comparison, the EAE (black bars) DRG levels of CX3CR1 protein are significantly increased over NC and AC animals at day 12 (EAE12 = 3.10 ± 0.54 ng/1 µg total protein, $P < 0.05$ and $P < 0.01$); however, EAE animals at days 3, 6, 9 and 15 do not show an increase over baseline (EAE3 = 2.20 ± 0.22 ng/1 µg total protein; EAE6 = 2.42 ± 0.17 ng/1 µg total protein; EAE9 = 2.65 ± 0.26 ng/1 µg total protein and EAE 15 = 2.62 ±

0.49 ng/1 μg total protein) as shown in **Figure 3(d)**. (ANOVA followed byTukey's posthoc test).

3.4.7. CX3CL1 GENE EXPRESSION ANALYSIS IN THE SC

CX3CL1 mRNA expression in the SC at different times following EAE induction is shown in **Figure 4(a)**. Results are shown as a ratio of CX3CL1 mRNA to GAPDH mRNA. NC animals (white bars) show CX3CL1 mRNA expression at 0.1054 ± 0.0131. AC animals (grey bars) shown a similar mRNA expression of CX3CL1 to that of NC animals at all time points (AC3 = 0.1111 ± 0.0153; AC6 = 0.1301 ± 0.0264; AC9 = 0.1768 ± 0.0092; AC12 = 0.1160± 0.0058; AC15 = 0.1270 ± 0.0209). However, EAE animals (black bars) showed a significant increase of CX3CL1 expression in the SC compared to NC animals at days 9, 12 and 15 (EAE9 = 0.1727 ± 0.026, $P < 0.01$; EAE12 = 0.2067 ± 0.0210, $P < 0.005$ and EAE15 = 0.1783 ± 0.0053, $P < 0.005$). However, no significant change of CX3CL1 expression was identified between EAE and NC animals at days 3 and 6 (EAE3 = 0.1253 ± 0.0059 and EAE6 = 0.1083 ± 0.0052). In addition, EAE animals also showed significant increases in mRNA expression over AC group at days 12 and 15 ($P < 0.005$ and $P < 0.05$, respectively) as shown in **Figure 4(a)**. (ANOVA followed by Tukey's posthoc test).

3.4.8. CX3CL1 PROTEIN EXPRESSION ANALYSIS IN THE SCBY ELISA

Total CX3CL1 protein expression in the SC is significantly altered at different times following EAE induction as shown in **Figure 4(b)**. Results are given as ng CX3CL1 per 10 µg total protein for each sample. NC animals (white bars) show a baseline level of CX3CL1 in the SC of 40.05 ± 6.09 ng/10 µg total protein. AC animals (grey bars) show a similar expression level of CX3CL1 to that of NC animals at all time points assessed (AC3 = 47.59 ± 8.33 ng/10 µg total protein; AC6 = 51.45 ± 7.30 ng/10 µg total protein; AC9 = 59.55 ± 8.97 ng/10 µg total protein; AC12 = 57.79 ± 2.62 ng/10 µg total protein and AC15 = 62.67 ± 9.61 ng/10 µg total protein). In comparison, the EAE (black bars) SC levels of CX3CL1 are significantly increased over NC baseline levels at days 6, 9 and 12 (EAE6 = 68.4 ± 9.16 ng/10 µg total protein, $P < 0.01$; EAE9 = 65.06 ± 7.29 ng/10 µg total protein, $P < 0.05$ and EAE12 = 93.61 ± 29.61 ng/10 µg total protein, $P < 0.005$), however, days 3 and 15 do not show a significant increase over NC baseline levels (EAE3 = 64.74 ± 2.11 ng/10 µg total protein and EAE 15 = 63.20 ± 14.76 ng/10 µg total protein). In addition, EAE animals SC levels of CX3CL1 protein are significantly increased over AC animals at day 12 (EAE12 = 93.61 ± 29.61 ng/10 µg total protein, $P < 0.005$), however, at days 3, 6, 9 and 15 is no significant increase over AC animals as shown in **Figure 4(b)**. (ANOVA followed by Tukey's posthoc test).

3.4.9. CX3CR1 GENE EXPRESSION ANALYSIS IN THE SC

The real time RT-PCR results show that CX3CR1 mRNA expression in the SC is not significantly altered at different times following EAE induction as shown in **Figure 4(c)**. NC animals (white bars) show CX3CR1 mRNA expression at 0.0398 ± 0.0061. AC animals (grey bars) at days 3, 6, 9, 12 and 15 (AC3 = 0.0424 ± 0.0036; AC6 = 0.0450 ± 0.0023; AC9 = 0.0443 ± 0.0041; AC12 = 0.0468 ± 0.0039 and AC15 = 0.0377 ± 0.0054) show a similar mRNA expression of CX3CR1 to that of NC animals. In comparison, EAE animals (black bars) at days 9, 12 and 15 (EAE9 = 0.0542 ± 0.0029, $P < 0.05$, EAE12 = 0.0630 ± 0.079, $P < 0.005$ and EAE15 = 0.0536 ± 0.0015) show a significant increase of CX3CR1 expression in SC compared to that of NC animals. However, there is no significant change of CX3CR1 expression in EAE animals at days 3, 6 and 9 (EAE3 = 0.0433 ± 0.0041, EAE6 = 0.0462 ± 0.0120 and EAE9 = 0.0542 ± 0.0029) when compared to that of AC animals at the same time points. Furthermore, EAE animals at days 12 and 15 show significant increase in mRNA expression over AC animals at the same time points ($P < 0.005$ and $P < 0.05$ respectively) as shown in **Figure 4(c)**. (ANOVA followed by Tukey's posthoc test).

3.4.9. CX3CR1 PROTEIN EXPRESSION ANALYSIS IN THE SC BY ELISA

Total CX3CR1 protein expression in the SC at different time points following EAE induction are shown in **Figure 4(d)**. Results are given as ng CX3CR1 per 1 µg total protein for each sample. NC animals (white bars) show a baseline level of CX3CR1 in

the SC of 2.20 ± 0.54 ng/1 µg total protein. AC animals (grey bars) show a similar expression level of CX3CR1 to that of NC animals at all time points assessed (AC3 = 2.03 ± 0.12 /1 µg total protein; AC6 = 2.19 ± 0.38 ng/1 µg total protein; AC9 = 1.82 ± 0.36 ng/1 µg total protein; AC12 = 1.94 ± 0.26 ng/1 µg total protein and AC15 = 2.50 ± 0.53 ng/10 µg total protein). EAE (black bars) SC levels of CX3CR1 protein are significantly increased over NC and AC animals at day 12 (EAE12 = 3.56 ± 1.29 ng/1µg total protein, $P < 0.05$ and $P < 0.005$) , however, days 3, 6, 9 and 15 do not show a significant increase in protein over NC and AC baseline protein expression levels (EAE3 = 2.13 ± 0.21 ng/1 µg total protein; EAE6 = 2.02 ± 0.19 ng/1 µg total protein; EAE9= 2.01 ± 0.36 ng/1 µg total protein and EAE15 = 2.41 ± 0.53 ng/1 µg total protein) as shown in **Figure 4(d)**. (ANOVA followed by Tukey's posthoc test).

3.4.10. IHC ANALYSIS OF CX3CL1 PROTEIN EXPRESSION IN THE SC

Expression of CX3CL1 and its receptor, CX3CR1, at EAE12, show immunoreactivity in the SC grey matter in different cell types **(Figures 5 and 6).** Specifically, CX3CL1 (red labeling) was found in neurons (NeuN: green labeling) as shown by double labeling of CX3CL1 with NeuN [yellow labeling, white arrow in **Figure 5(a)**. Likewise, CX3CL1 was expressed in glial cells, as co-localization of CX3CL1 with GFAP (astrocyte marker: green labeling) was observed [yellow labeling, white arrows in **Figure 5(b)** in EAE12 rats.

3.4.11. IHC CX3CR1 PROTEIN EXPRESSION IN THE SC

Expression of CX3CR1 (red labeling) in neurons (NeuN: green labeling), was confirmed by co-localization of CX3CR1 with NeuN [yellow labeling, white arrow in **Figure 6(a)** which was predominantly concentrated in the dorsal horn of SC (*asterisk). As shown by co-localization of CX3CR1 (red labeling) with CD68 (macrophage marker: green labeling), CX3CR1 is expressed in microglia [yellow labeling, white arrow in **Figure 6(b)**. Images were taken at a total magnification of 100X (top panels) and 400X (bottom panels) from EAE12 rats.

3.4.12. IHC ANALYSIS OF CX3CR1 PROTEIN EXPRESSION IN THE SC

Expression of CX3CR1 (red labeling) in neurons (NeuN: green labeling), was confirmed by co-localization of CX3CR1 with NeuN (yellow labeling, white arrow in **Figure 6**(**a**) which was predominantly concentrated in the dorsal horn of SC (*asterisk). As shown by co-localization of CX3CR1 (red labeling) with CD68 (macrophage marker: green labeling), CX3CR1 is expressed in microglia (yellow labeling, white arrow in **Figure 6**(**b**). Images were taken at a total magnification of 100X (top panels) and 400X (bottom panels) from EAE12 rats.

3.5. DISCUSSION

MS is an autoimmune disease whose pathology involves many of the same inflammatory mediators [tumor necrosis factor alpha (TNF α)] that are also commonly associated with the development of chronic pain syndromes such as NPP [5, 37]. The EAE rat model of MS–induced NPP is an ideal model to identify the early molecular mechanisms underlying the pathophysiology of NPP because this model characteristically induces immune system-mediated inflammation without demyelination [26, 27]. As a result, this NPP model allows for the identification of molecular changes in pain induction from the earliest onset of an initial immune system-mediated inflammatory event known to induce NPP prior to any de-myelination.

NPP has been reported as the second worst disease induced symptom reported to occur in up to 75% of patients with MS [1, 38]. Interestingly, NPP has also reported to be present in MS patients prior to the time of diagnosis and therefore may be a promising pre-diagnostic indicator to facilitate the early diagnosis of MS [39].

Previous studies have also shown that EAE animals experience NPP as part of their immune system-mediated disease progression [36]. CX3CL1 and CX3CR1 are established factors in the modulation of pain perception via a central pro-algesic mechanism [40]. In our study, we demonstrated using an EAE model of MS-induced NPP significant changes in CX3CL1 and its receptor CX3CR1 in the DRG and SC. Specifically, our study confirms that CX3CL1 expression is increased in the DRG and SC

during the early inflammatory phase of EAE induction prior to the demyelination. As a result, this study confirms the importance of the immune system in pain induction prior to any detectable tissue damage or injury. Interestingly, the increased expression of CX3CL1 directly correlates with the behavioral data that confirms thermal hypoalgesia (a sensory abnormality known to occur in NPP prior to hyperalgesia [36]). As a result, our molecular and behavioral findings suggest that CX3CL1 is a nociceptive mediator induced in the early stages of inflammation by the immune system prior to any detection of myelin damage or injury. Henceforth, CX3CL1 is a nociceptive mediator involved in the early induction of immune system-mediated MS–induced NPP.

NPP is a chronic pain syndrome that has been associated with abnormal sensory changes in response to mechanical, chemical or thermal stimuli. Due to the variability by which NPP presents, it is unlikely to display sensory abnormalities in response to three forms of stimuli at once. Our results are consistent with the literature in this regard, as our EAE animals only displayed sensory abnormalities to thermal rather than mechanical stimuli. Our study confirms the importance of testing for all three forms of sensory stimuli to ensure all subdomains of NPP are properly tested to confirm its presence or absence in the models being tested.

CX3CL1 is the only member of the fourth group of chemokines with the CX3C motif. It exists in two forms: membrane-bound, tethered to the cell membrane by a mucin-like stalk, and as a soluble protein following cleavage [10]. CX3CL1 is constitutively expressed by neurons in the brain, SC, and DRG [20, 21]. Under normal physiological

conditions, membrane-bound CX3CL1 is cleaved by ADAM17 [a matrix metalloproteinase formerly known as TNF converting enzyme (TACE)] to release soluble CX3CL1 [41]. In inflammatory states, increased expression of CX3CL1 occurs in neurons and also in astrocytes in the dorsal horn of the SC [13]. Interestingly, peripheral nerve injury results in a decrease in membrane-bound CX3CL1 within DRG neurons [16], but not in the dorsal horn of the SC [13, 21]. The CX3CL1 receptor, CX3CR1, is constitutively expressed in microglia of the brain and SC [13, 19], and is significantly increased as a result of microglial activation [12, 21]. CX3CR1 is known to be critical for the generation of NPP, as mice lacking CX3CR1 do not develop allodynia following peripheral nerve injury [42]. As a result, the results from our research also support the role of CX3CL1 and its receptor CX3CR1 in the induction of MS-induced NPP. Furthermore, our results also support the importance of the key anatomical connection between the DRG and SC as an integral molecular signaling pathway for which CX3CL1 can exerts its pathological effects associated with the induction of NPP through its CX3CR1 receptor. Although we used antigenic induction of the immune system to elicit an inflammatory response to trigger the behavioral changes consistent with NPP, other research using noxious electrical stimulation also linked CX3CL1 to the induction, amplification, and maintenance of injury induced pain [43].

Following inflammation, injured neurons release adenosine-5'-triphosphate (ATP), which binds to the P2X7 receptor on microglia, that subsequently causes the release of the protease Cathepsin S [44]. CX3CL1 is bound to the neuronal membrane, and is cleaved by the Cathepsin S [45]. Soluble CX3CL1 binds to the CX3CR1 on microglia resulting in

the increased synthesis and release of pro-nociceptive mediators, such as IL-6 and nitric oxide [24]. These pro-nociceptive mediators bind to receptors on dorsal horn SC neurons, resulting in enhanced hypersensitivity and spontaneous firing that characterize central pain [24] which subsequently creates a positive feedback loop that pathogenically maintains the CX3CL1/CX3CR1 signaling pathway.

Early studies have shown that CX3CL1 and CX3CR1 play important roles in neuron-glia communication [43]. In our previous published studies [5, 27], we showed significantly the up-regulation of the proinflammatory cytokine TNF α at the gene and protein levels within the DRG and SC in the EAE model of MS. Recent research has shown that TNF α induces CX3CL1 expression in endothelial cells [40]. Furthermore, TNF α also has functional implications in the post-transcriptional regulation of CX3CL1 [46, 47]. These findings indicate that TNF α is a critical upstream factor that regulates CX3CL1 production. Thus, our previously published studies in this area suggest that up-regulation of TNF α in the DRG and SC may be a critical early step in regard to the regulation of MS pain induction via CX3CL1/CX3CR1 signaling pathway. However, further studies are required to definitively confirm this molecular signaling link between TNF α and CX3CL1. Additional studies are also required to study the effect of TNF α on CX3CL1 expression in SC neurons, astrocytes and microglia.

Our data shows significant changes in CX3CL1 and its receptor CX3CR1 at both gene and protein levels within the DRG and SC. Interestingly, these changes also correlated with the onset and peak NDS. Henceforth, we conclude that the changes in CX3CL1 and

CX3CR1 expression levels are the direct result of activation of the immune response by CNS-myelin-specific antigens, such as MBP. Based on our experimental findings, we also propose that CX3CL1 may be involved in the molecular signaling cascade that ultimately contributes to myelin damage and subsequent neurological disability associated with MS. However, further investigation in this concept needs to be conducted before any definitive conclusions can be drawn in this regard as the events identified in an EAE animal model do not always directly reflect those events which occur in humans with MS. Irrespectively, it is our belief that the CX3CL1/CX3CR1 signaling crosstalk between neurons and microglia in the SC may be involved in the underlying pathology associated with MS [43]. Additional research also supports the concept of CX3CL1/CX3CR1 involvement in the pathogenesis of MS. For example, researchers have linked CX3CL1 to the recruitment of NK cells that modify EAE within the CNS [48]. Furthermore, others have demonstrated that blockade of CX3CL1 protected mice against EAE [49]. However, it is still unclear how the synthesis and secretion of CX3CL1 are regulated and which pathways of CX3CL1 signaling in glia cells are utilized to exert these effects.

Our study demonstrated that in an inflammatory state, CX3CL1 is expressed in neurons and astrocytes, and its receptor CX3CR1 is expressed predominantly in microglia. A remarkable finding of our study is that in the EAE model, CX3CL1 and its receptor (CX3CR1) show significant changes in expression pattern that correlates with the onset of sensory abnormalities, indicating that the activation of glial cells by an inflammatory response leads to increased pain signaling between neurons and microglia. Our finding

that CX3CR1 immunoreactivity is localized on microglia and dorsal horn SC neurons is interesting because CX3CR1 is usually expressed on microglia in the CNS [40]. Thus our findings indicate that neuronal expression of CX3CR1 occurs as a direct result of CNS inflammation. This expression change of CX3CR1 in SC neurons may be a critical mechanism involved in MS–induced NPP. Further, our study demonstrated that CX3CL1 is expressed in neurons, but its receptor CX3CR1 is expressed predominately in microglia. This finding is in concordance with previously published studies showing that CX3CL1 works as a molecule signaling from neuron to microglia to induce glia activation. SC microglia and astrocytes have been shown to be implicated in various types of NPP such as peripheral nerve injury, bone cancer and spinal root constriction [25, 50], thereby confirming that glia activation is directly involved in inflammation and NPP. Recent research has shown that administration of glial inhibitors exerts anti-allodynic function [51, 52]. In our research, we found that astrocytes also express CX3CL1 in the EAE SC, suggesting that activated astrocytes are also involved in the induction of pain.

Our data suggests that during the early inflammatory stage of MS prior to demyelination, CX3CL1 signaling in dorsal horn SC neurons activates the ascending pathways involved in nociceptive transmission. Interestingly, increased serum levels of CX3CL1 have been reported to be seen in MS patients without significant changes in CX3CL1 levels in the cerebral spinal fluid (CSF) [53]. Studies are ongoing, to investigate the correlation between serum levels of CX3CL1 in patients with relapsing remitting MS, at different stages of their disease (relapse vs stable remission phase). Henceforth, our study suggests

that CX3CL1 and/or its receptor CX3CR1 may be easily assessed biomarkers of MS-induced NPP.

3.6. CONCLUSION

Previous research into chronic pain, predominantly identified drug, injury or disease induced causes of pain without fully considering the impact of the immune system to the contribution in chronic pain development. Henceforth, current research has now placed a greater emphasis on the early aspects of pain induction by exploring the neuroimmune modulation of the pain response [5]. In our study we used an MBP animal model of MS that depicts immune system activation of inflammation without demyelination. As such, this EAE model represents an ideal model to study the molecular changes that occur at the earliest stages of immune system activation prior to any tissue damage. The ability to target the molecular changes occurring at the earliest phase of pain induction following immune system activation could minimize the involvement of the downstream nociceptive mediators involved in the induction and/or maintenance of chronic pain. Previous research has shown that TNF α induces CX3CL1 expression [40] and that it has functional implications in the post-transcriptional regulation of CX3CL1 [46, 47]. Our current findings for CX3CL1 expression correlate closely with our previous published studies on TNF α, thereby supporting the concept that TNF α is an integral factor associated with the induced production of CX3CL1. Taken together, these studies suggest that the immune mediated up-regulation of TNF α in the DRG and SC may be a critical upstream signaling pathway that regulates MS pain induction by governing CX3CL1

expression. However, further studies are required to definitively confirm this molecular signaling link between TNF α and CX3CL1. Furthermore, our research also showed significant elevations in the expression of CX3CL1 and its receptor CX3CR1 at both the gene and protein levels within the DRG and SC that correlated with the behavioral data suggestive of NPP. As a result, novel therapeutic interventions aimed at blocking CX3CR1 may prove to be beneficial in attenuating sensory abnormalities associated with neuropathies associated with CX3CL1 induction. In addition, our findings confirmed the expression changes in CX3CL1 and CX3CR1 to occur within the DRG and SC. As a result, our research confirms the importance of the key anatomical connection between the DRG and SC via the connecting dorsal roots as being a critical pathway for the upstream nociceptive molecular signaling of CX3CL1/CX3CR1 following neuroimmune activation. Our findings also confirmed that neurons and astrocytes in the SC express CX3CL1 while neurons and microglia express CX3CR1. As a result, our findings are consistent with other researchers that support the involvement of microglia activation in the SC in regard to the induction and/or maintenance of chronic NPP [54]. However, additional studies are required to determine the effect of TNF α on CX3CL1 expression in SC neurons, astrocytes and microglia. In summary, our EAE study results suggest that CX3CL1 and its receptor CX3CR1 may be suitable easily assessed biomarkers of MS-induced NPP that could assist clinicians in the diagnosis and early treatment intervention of MS–induced NPP.

3.7. TABLES

Tail:	**Bladder:**
0 – normal	0 – normal
1 – partially paralyzed, weakness	1 - incontinence
2 – completely paralyzed, limp	

Right hind limb:	**Left hind limb**:
0 – normal	0 – normal
1 – weakness	1 - weakness
2 – dragging with partial paralysis	2 - dragging with partial paralysis
3 – complete paralysis	3 – complete paralysis

Right forelimb:	**Left forelimb:**
0 – normal	0 - normal
1 – weakness	1 - weakness
2 – dragging, not able to support weight	2 – dragging, not able to support weight
3 – complete paralysis	3 – complete paralysis

Table 1: Neurological disability clinical scoring system for animals induced to a state of EAE. The total score is the sum of the following individual scores obtained for each of the 6 specified clinical domains. Following induction, each rat was assessed twice daily for clinical signs of EAE as previously described [27], thereby rendering an average daily mean score. EAE animals after disease onset were assessed three times daily.

thereby rendering an average daily mean score. Daily body weight and hydration status was also measured to assess general animal health and well-being.

3.8. FIGURES

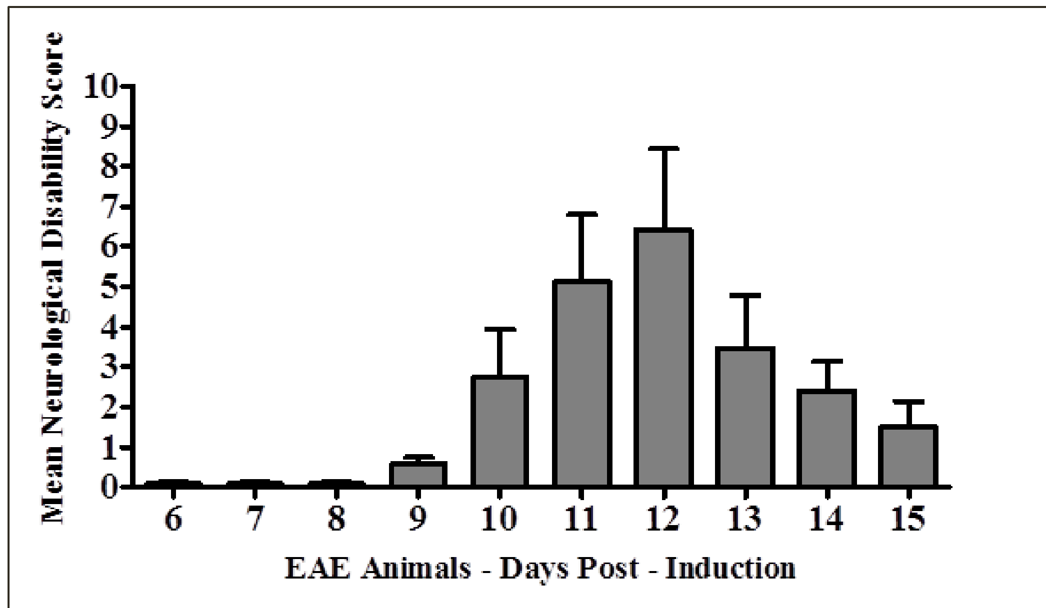

Figure 1: EAE animals neurological disability clinical score. All animals in the EAE group were assessed for neurological disability according to a previously described global neurological disability assessment tool [26, 27] detailed in **Table 1**. Disability scores range from a mean clinical disability score of 0 (no disability) to 15 (maximum disability). The bell shaped distribution outlining peak neurological disability in response to EAE induction occurred at EAE12. Clinical neurological deficits appear at 6 days after antigenic induction. By EAE9 all animals started to display clinical signs of neurological disability (0.57 ± 0.45; mean ± SEM). Neurological disability progressively worsened upon daily assessment until EAE12 (peak disability; 6.42 ± 5.35), then subsided by EAE15 (1.5 ± 1.41) as the animals entered the remission phase of disease induction, well characterized for this animal model. Errors bars represent SEM.

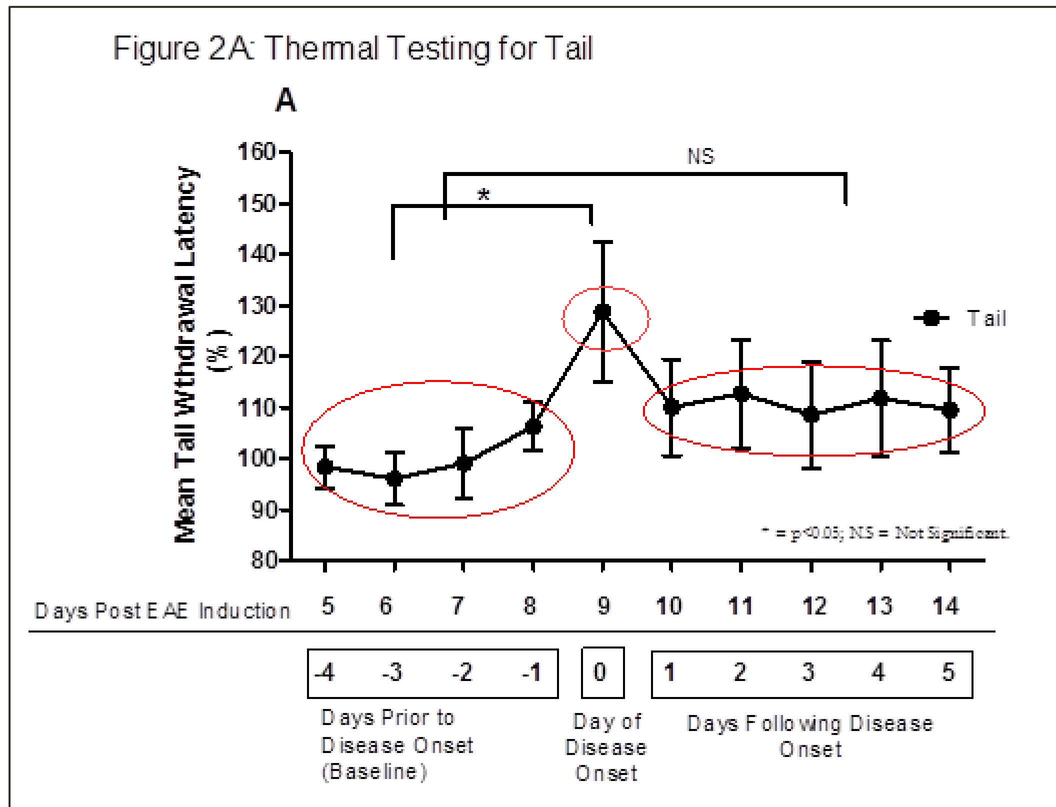

Figure 2(a): Thermal testing for tail in EAE animals. This figure illustrates withdrawal latencies of thermal sensory testing in the tails from EAE animals, at different times in the disease progression. Data are aligned on the "x axis" to day of onset of neurological disability, where day 0 (= EAE9) is the first day of disease onset (where all EAE animals displayed some form of neurological disability). For example day 5 is representative of 5 days following disease onset which equates to EAE14. However, days -4 to -1 (baseline) represent the days prior to the onset of neurological disability that equate to EAE5, EAE6, EAE7, and EAE8 days post-induction. All values were normalized to average baseline withdrawal latencies and displayed as means ± SEM. Tail withdrawal latency was significantly elevated at day 0 (day of disease onset = EAE9) compared to the average baseline withdrawal latency, indicative of thermal hypoalgesia

in the EAE animals (*P = 0.023, using a one sample t-test). Following this peak at EAE9, the thermal latency for tail declined and remained stable over the time period of EAE10 to EAE14 days following induction. Withdrawal latencies at baseline were not statistically significantly different from that depicted for days 1, 2, 3, 4, and 5 after disease onset. Errors bars represent SEM.

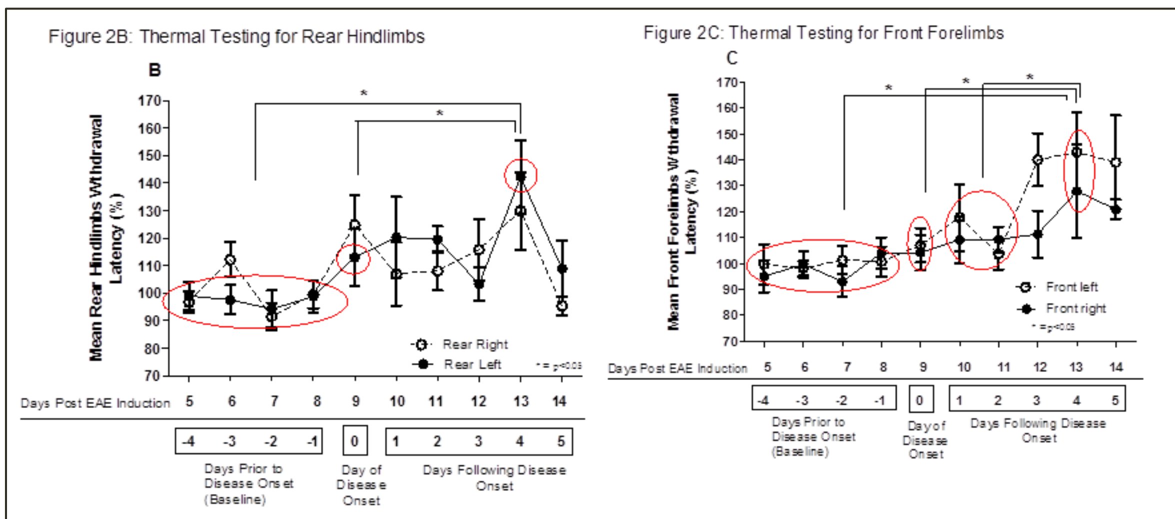

Figure 2(b-c): Thermal testing for hind limbs and forelimbs in EAE animals. Figures 2 b-c illustrate withdrawal latencies of thermal sensory testing in the hind limb and forelimb from EAE animals, at different times in the disease progression. Data are aligned on the "x axis" to day of onset of neurological disability, where day 0 (= EAE9) is the first day of disease onset (where all EAE animals displayed some form of neurological disability). For example day 5 is representative of 5 days following disease onset which equates to EAE14. However, days -4 to -1 (baseline) represent the days prior to the onset of neurological disability that equate to EAE5, EAE6, EAE7, and EAE8 days following induction. All values were normalized to average baseline withdrawal latencies

and displayed as means ± SEM. **(b)** A statistically significant increase (*$P < 0.05$) of the withdrawal latency characteristic of thermal hypoalgesia was observed in the left hind limb at day 4 following the onset of the disease compared to that of average baseline withdrawal latency (days -1 to -4 inclusive) and to day 0 (onset of neurological disability). **(c)** Similarly, a statistically significant increase (*$P < 0.05$) of the withdrawal latency characteristic of thermal hypoalgesia was observed in the right and left forelimbs at day 4 following the onset of the disease compared to average baseline withdrawal latency (days -1 to -4 inclusive); day 0 (onset of neurological disability); and days 1 and 2 after disease onset. Errors bars represent standard error of the mean SEM.

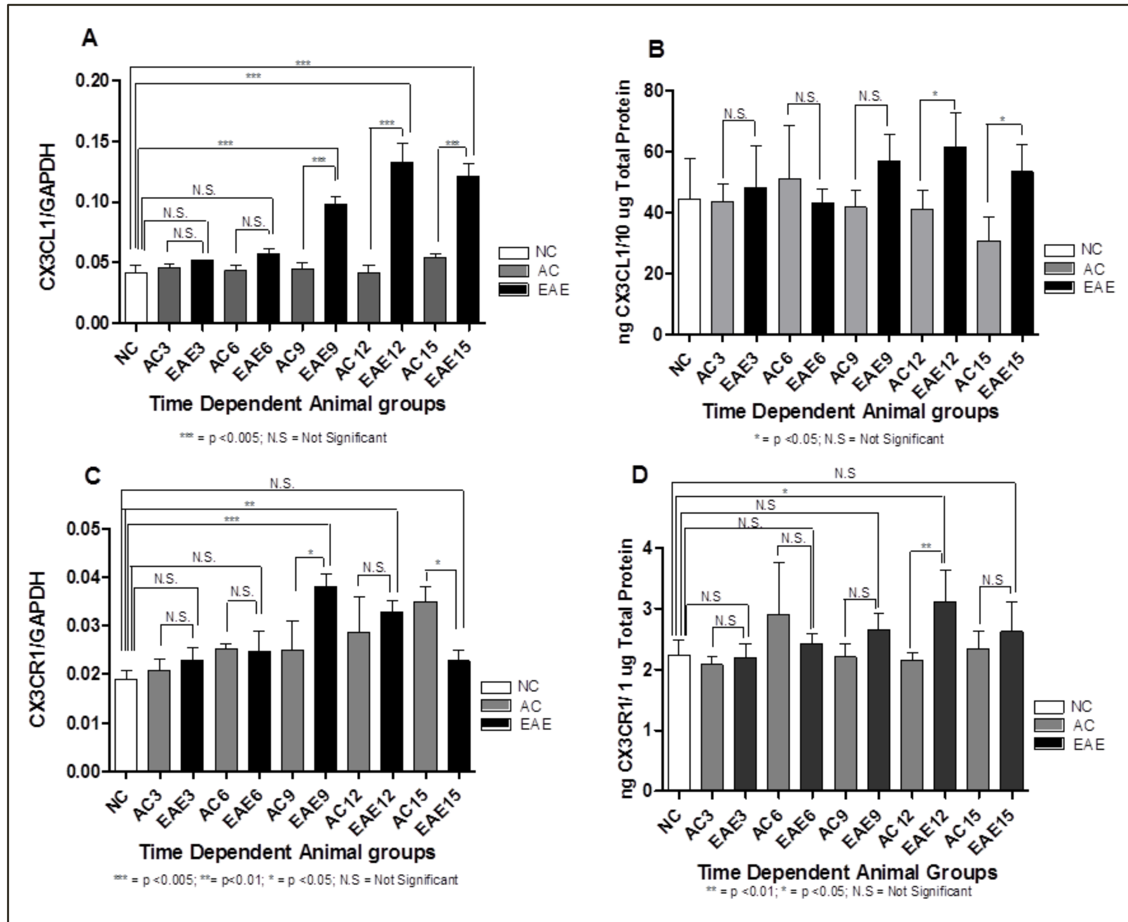

Figure 3 (a-d): Gene and protein expression of CX3CL1 and its receptor CX3CR1 in DRG. **(a)** This figure illustrates CX3CL1 mRNA gene expression in the DRG, at different times in the disease progression. Naïve control (NC) animals (white bars) show CX3CL1 mRNA expression at 0.0419 ± 0.0058. AC animals (grey bars) shown a similar mRNA expression of CX3CL1 to NC at all-time points (AC3 = 0.0456 ± 0.0028; AC6 = 0.0434 ± 0.0041; AC9 = 0.0449 ± 0.0047; AC12 = 0.0416 ± 0.0065; AC15 = 0.0540 ± 0.0035). In comparison, EAE animals (black bars) show a significant increase of CX3CL1 expression in DRG over NC at days 9, 12 and 15 post-induction (EAE9 = 0.0983 ± 0.0065, $P < 0.005$; EAE12 = 0.1323 ± 0.0154, $P < 0.005$ and EAE15 = 0.1208 ±

0.0102, $P < 0.005$). Furthermore, EAE animals show significant increase in mRNA expression over AC group at days 9, 12 and 15 ($P < 0.005$). However, there is no significant change of CX3CL1 expression between EAE and NC at days 3 and 6 (EAE3 = 0.0520 ± 0.0006 and EAE6 = 0.0575 ± 0.0045). (***$P < 0.005$; ns = not significant; ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(b)** This figure illustrates CX3CL1 protein expression in the DRG, at different times in the disease progression. NC animals (white bars) show CX3CL1 protein expression at 44.42 ± 13.58 ng/10 µg of total protein. AC animals (grey bars) shown a similar protein expression of CX3CL1 to NC at all-time points (AC3 = 43.85 ± 5.54 ng/10 µg total protein; AC6 = 51.22 ± 17.60 ng/10 µg total protein; AC9 = 41.86 ± 5.43 ng/10 µg total protein; AC12 = 41.25 ± 6.31 ng/10 µg total protein; AC15 = 30.87 ± 7.77 ng/10 µg total protein). In comparison, EAE animals (black bars) show a significant increase of CX3CL1 expression in DRG over AC at days 12 and 15 (EAE12 = 61.74 ± 10.98 ng/10 µg total protein, $P < 0.05$ and EAE15 = 53.48 ± 8.87 ng/10 µg total protein, $P < 0.05$). However, there is no significant change of CX3CL1 expression between EAE and AC at days 3, 6 and 9 (EAE3 = 48.18 ± 13.79 ng/10 µg total protein; EAE6 = 43.44 ± 4.33 ng/10 µg total protein and EAE9 = 56.82 ± 8.87 ng/10 µg total protein). (*$P < 0.05$; ns = not significant, ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(c)** This figure illustrates CX3CR1 mRNA gene expression in DRG at different times in the disease progression. NC animals (white bars) show CX3CR1 mRNA expression at 0.0189 ± 0.0019. AC animals (grey bars) shown a similar mRNA expression of CX3CR1 to NC at days 3, 6, 9 and 12 (AC3 = 0.0208 ± 0.0023; AC6 = 0.0254 ± 0.0009; AC9 = 0.0249 ± 0.0060; AC12 = 0.0287 ± 0.0073)

except for the significant change at day 15 (AC15 = 0.0349 ± 0.0032, $P < 0.01$). In comparison, EAE animals (black bars) show a significant increase of CX3CR1 expression in DRG over NC at days 9 and 12 (EAE9 = 0.0379 ± 0.0028, $P < 0.005$ and EAE12 = 0.0327 ± 0.0252, $P < 0.01$). However, there is no significant change of CX3CR1 expression between EAE and NC at days 3, 6 and 15 (EAE3 = 0.0228 ± 0.0026, EAE6 = 0.0248 ± 0.0041 and EAE15 = 0.0228 ± 0.0022). However, EAE animals did show a significant increase in mRNA expression over AC group at days 9 and 15 ($P <$ 0.05 and $P < 0.05$ respectively). (*$P < 0.05$; ** $P < 0.01$; ***$P < 0.005$; ns = not significant; ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(d)** This figure illustrates CX3CR1 protein expression in the DRG at different times in the disease progression. NC animals (white bars) show a baseline level of CX3CR1 in the DRG of 2.24 ± 0.24 ng/1 μg total protein. AC animals (grey bars) show a similar expression level of CX3CR1 compared to that of NC animals at all time points assessed (AC3 = 2.08 ± 0.15 ng/1 μg total protein; AC6 = 2.90 ± 0.85 ng/1 μg total protein; AC9 = 2.21 ± 0.22 ng/1 μg total protein; AC12 = 2.15 ± 0.13 ng/1 μg total protein and AC15 = 2.34 ± 0.29 ng/1 μg total protein). In comparison, the EAE (black bars) DRG levels of CX3CR1 protein are significantly increased over NC and AC animals at day 12 (EAE12 = 3.10 ± 0.54 ng/1 μg total protein, $P < 0.05$ and $P < 0.01$) , however EAE animals at days 3, 6, 9 and 15 do not show an increase over baseline (EAE3 = 2.20 ± 0.22 ng/1 μg total protein; EAE6 = 2.42 ± 0.17 ng/1 μg total protein; EAE9 = 2.65 ± 0.26 ng/1 μg total protein and EAE 15 = 2.62 ± 0.49 ng/1 μg total protein) as shown in **Figure 3(d)**. (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; ns = not significant,

ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD).

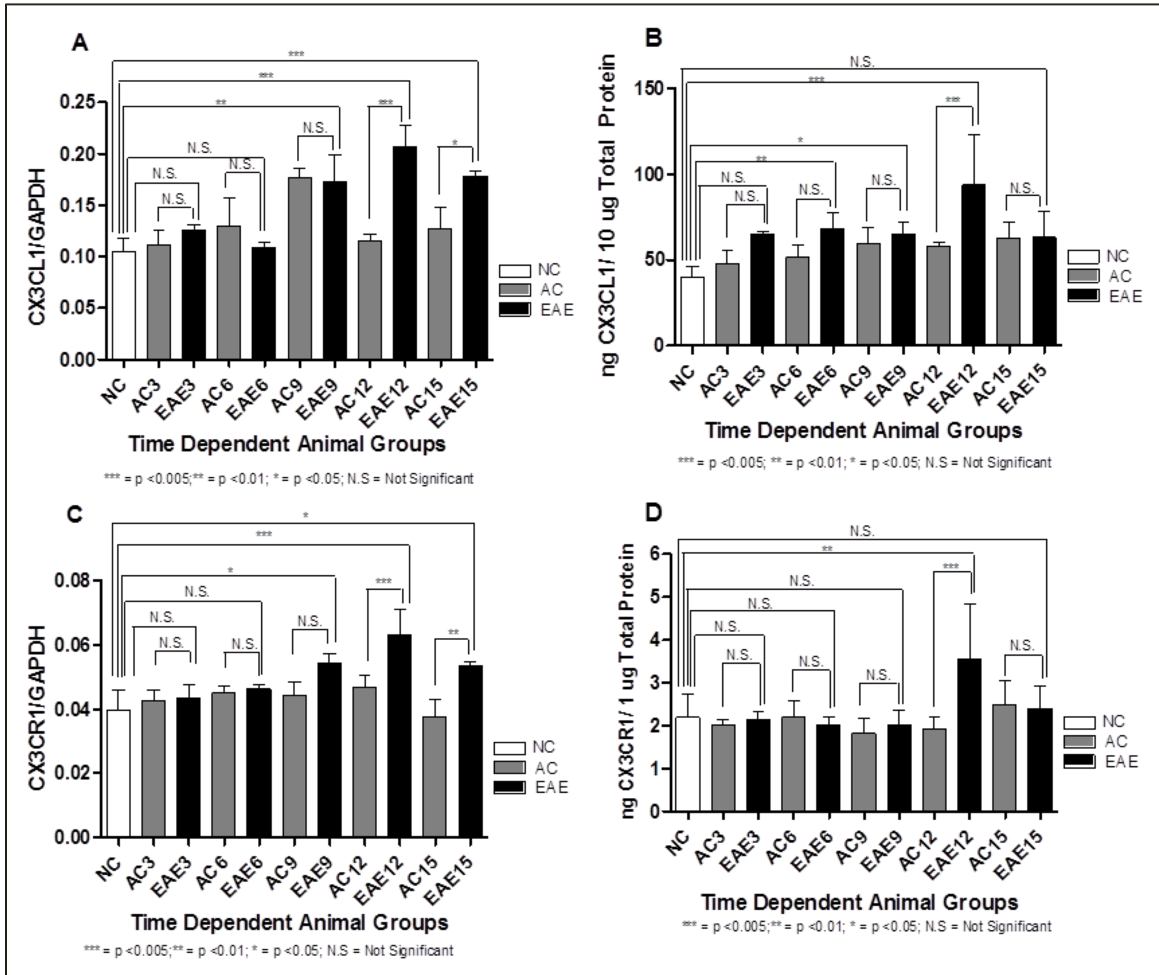

Figure 4 (a-d): Gene and protein expression of CX3CL1 and its receptor CX3CR1 in SC. (a) This figure illustrates CX3CL1 mRNA gene expression in the SC at different times in the disease progression. NC animals (white bars) show CX3CL1 mRNA expression at 0.1054 ± 0.0131. AC animals (grey bars) shown a similar mRNA expression of CX3CL1 to that of NC animals at all time points (AC3 = 0.1111 ± 0.0153; AC6 = 0.1301 ± 0.0264; AC9 = 0.1768 ± 0.0092; AC12 = 0.1160 ± 0.0058; AC15 =

0.1270 ± 0.0209). However, EAE animals (black bars) showed a significant increase of CX3CL1 expression in the SC compared to NC animals at days 9, 12 and 15 (EAE9 = 0.1727 ± 0.026, $P < 0.01$; EAE12= 0.2067 ± 0.0210, $P < 0.005$ and EAE15 = 0.1783 ± 0.0053, $P < 0.005$). However, no significant change of CX3CL1 expression was identified between EAE and NC animals at days 3 and 6 (EAE3 = 0.1253 ± 0.0059 and EAE6 = 0.1083 ± 0.0052). In addition, EAE animals also showed significant increases in mRNA expression over AC group at days 12 and 15 ($P < 0.005$ and $P < 0.05$ respectively). (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; ns = not significant, ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(b)** This figure illustrates CX3CL1 protein expression in the SC, at different times in the disease progression. NC animals (white bars) show a baseline level of CX3CL1 in the SC of 40.05 ± 6.09 ng/10 μg total protein. AC animals (grey bars) show a similar expression level of CX3CL1 to that of NC animals at all time points assessed (AC3 = 47.59 ± 8.33 ng/10 μg total protein; AC6 = 51.45 ± 7.30 ng/10 μg total protein; AC9 = 59.55 ± 8.97 ng/10 μg total protein; AC12 = 57.79 ± 2.62 ng/10 μg total protein and AC15 = 62.67 ± 9.61 ng/10 μg total protein). In comparison, the EAE (black bars) SC levels of CX3CL1 are significantly increased over NC baseline levels at days 6, 9 and 12 (EAE6 = 68.4 ± 9.16 ng/10 μg total protein, $P < 0.01$; EAE9 = 65.06 ± 7.29 ng/10 μg total protein, $P < 0.05$ and EAE12 = 93.61 ± 29.61 ng/10 μg total protein, $P < 0.005$), however, days 3 and 15 do not show a significant increase over NC baseline levels (EAE3 = 64.74 ± 2.11 ng/10 μg total protein and EAE 15 = 63.20 ± 14.76 ng/10 μg total protein). In addition, EAE animals SC levels of CX3CL1 protein are significantly increased over AC animals at day 12 (EAE12 = 93.61 ± 29.61 ng/10 μg total protein, $P < 0.005$), however, at days 3,

6, 9 and 15 do not show a significant increase over AC animals. (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; ns = not significant, ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(c)** This figure illustrates CX3CR1 mRNA expression in the SC, at different times in the disease progression. NC animals (white bars) show CX3CR1 mRNA expression at 0.0398 ± 0.0061. AC animals (grey bars) at days 3, 6, 9, 12 and 15 (AC3 = 0.0424 ± 0.0036; AC6 = 0.0450 ± 0.0023; AC9 = 0.0443 ± 0.0041; AC12 = 0.0468 ± 0.0039 and AC15 = 0.0377 ± 0.0054) shown a similar mRNA expression of CX3CR1 to that of NC animals. In comparison, EAE animals (black bars) at days 9, 12 and 15 (EAE9 = 0.0542 ± 0.0029, $P < 0.05$, EAE12 = 0.0630 ± 0.079, $P < 0.005$ and EAE15 = 0.0536 ± 0.0015) show a significant increase of CX3CR1 expression in SC compared to that of NC animals. However, there is no significant change of CX3CR1 expression in EAE animals at days 3, 6 and 9 (EAE3 = 0.0433 ± 0.0041, EAE6 = 0.0462 ± 0.0120 and EAE9 = 0.0542 ± 0.0029) when compared to that of AC animals at the same time points. Furthermore, EAE animals at days 12 and 15 show significant increase in mRNA expression over AC animals at the same time points ($P < 0.005$ and $P < 0.05$ respectively). (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; ns = not significant, ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD). **(d)** This figure illustrates CX3CR1 protein expression in the SC, at different times in the disease progression. NC animals (white bars) show a baseline level of CX3CR1 in the SC of 2.20 ± 0.54 ng/1 µg total protein. AC animals (grey bars) show a similar expression level of CX3CR1 to NC animals all time points assessed (AC3 = 2.03 ± 0.12 /1 µg total protein; AC6 = 2.19 ± 0.38 ng/1 µg total protein; AC9 = 1.82 ± 0.36 ng/1 µg total protein; AC12 = 1.94 ± 0.26 ng/1 µg total protein and AC15 = 2.50 ±

0.53 ng/10 µg total protein). However EAE (black bars) SC levels of CX3CR1 protein are significantly increased over NC and AC animals at day 12 (EAE12 = 3.56 ± 1.29 ng/1µg total protein, $P < 0.05$ and $P < 0.005$), however, days 3, 6, 9 and 15 do not show a significant protein increase over NC and AC baseline protein expression levels (EAE3 = 2.13 ± 0.21 ng/1 µg total protein; EAE6 = 2.02 ± 0.19 ng/1 µg total protein; EAE9= 2.01 ± 0.36 ng/1 µg total protein and EAE15 = 2.41 ± 0.53 ng/1 µg total protein). (**$P < 0.01$; *** $P < 0.005$; ns= not significant, ANOVA followed by Tukey's posthoc test). Errors bars represent standard deviation (SD).

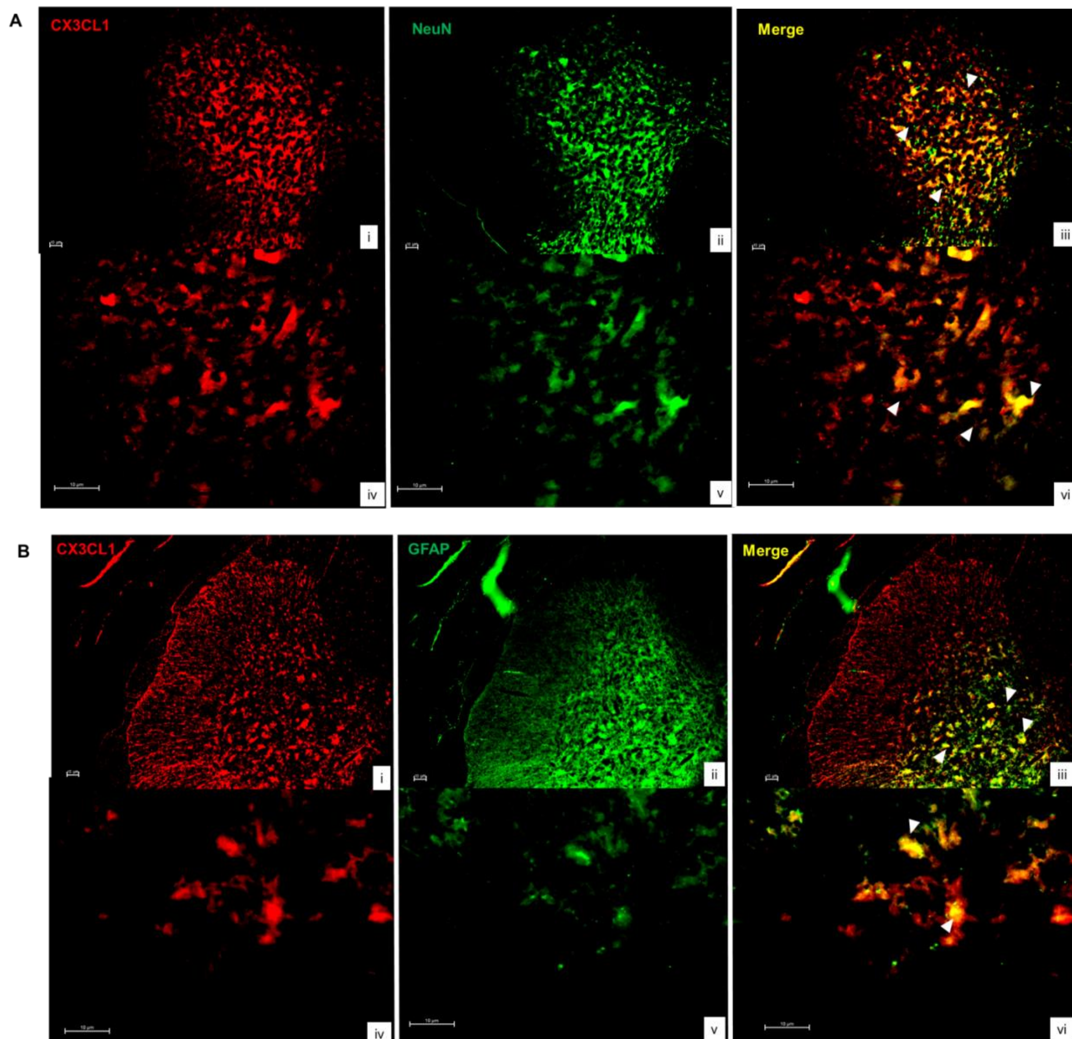

Figure 5(a-b): CX3CL1 expression in the SC of EAE12 rats. Double labeled immunofluorescence for CX3CL1 and markers for neurons (NeuN) or astrocytes (GFAP) in the SC of EAE12 rats. **(a)** CX3CL1 was expressed in neurons in the grey matter of SC. CX3CL1 (panels I & IV: red) in neurons (NeuN: panels II & V: green). CX3CL1 co-localizes with neurons (panels III & VI: yellow, arrows). **(b)** Co-localization between CX3CL1 and astrocytes was observed in the grey matter of SC. CX3CL1 (panels I & IV: red) in astrocytes (GFAP: panels II & V: green). CX3CL1 co-localizes with astrocytes

(panels III & VI: yellow, arrows). Images were taken at a total magnification of 100X (panels I-III) and 400X (panels IV-VI) from EAE 12 group. Scale bars = 10 µm.

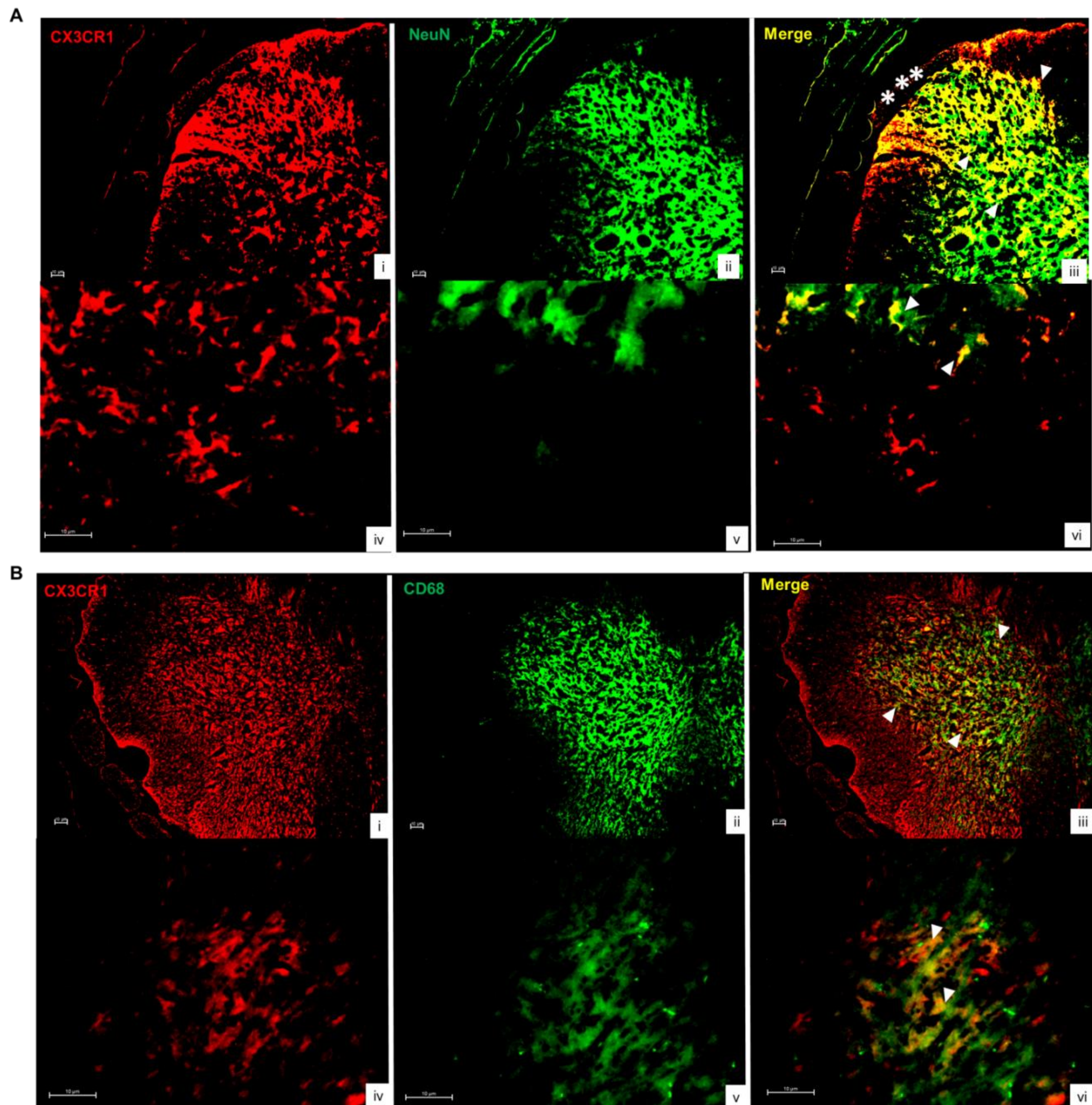

Figure 6(a-b): CX3CR1 expression in the SC of EAE12 rats. Double labeled immunofluorescence for CX3CR1 and markers for microglia (CD68) or neurons (NeuN) in the SC of EAE12 rats. **(a)** Co-localization between CX3CR1 and neurons was

observed in the dorsal horn (in asterisk) of SC. CX3CR1 (panels I & IV: red) in neurons (NeuN: panels II & V: green). CX3CR1 co-localizes with neurons (panels III & VI: yellow, arrows). **(b)** CX3CR1 was expressed in microglia in the grey matter of SC. CX3CR1 (panels I & IV: red) in microglia (CD68: panels II & V: green). CX3CR1 co-localizes with microglia (panels III & VI: yellow, arrows). Images were taken at a total magnification of 100X (panels I-III) and 400X (panels IV-VI) from EAE 12 group. Scale bars = 10 µm.

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PROLOGUE TO CHAPTER 4: RATIONALE FOR MANUSCRIPT

Previously, we showed that antigenic induction leads to significant increases in the expression of the neurotrophin, BDNF, in the DRG and SC in an animal model of MS [1]. Recent research has suggested a putative role for BDNF in the improvement of disease processes associated with MS [2-8]. The actions of BDNF are mediated through the TrkB receptor [9]. In the adult CNS, the expression of neurotrophin receptors varies according to tissue type and disease state. Therefore, we embarked on a study to examine the time dependent changes in TrkB expression in the DRG and SC in an inflammatory animal model of MS. By investigating the expression patterns and cellular source of BDNF during EAE we can better elucidate the role of BDNF during inflammatory processes contributing to myelin damage. Such insights would give us a better understanding of BDNF actions via TrkB that may be a target of novel therapies aimed at myelin repair or remyelination.

This study supports the role of DRG activation and BDNF signaling during disease induction. By determining patterns of BDNF signaling via TrkB, this study indirectly supports a potential role of NGF during EAE. Since the literature suggests they have reciprocal effects on each other [10-13], an increase in BDNF signaling should have an associated increase in NGF expression. This study, therefore, raises questions regarding NGF that need to be explored such as its expression pattern throughout the disease course and how it is related to BDNF signaling. Correlating NGF and BDNF expression is important because the relationship between these two factors is a critical aspect of our

proposed signaling triad which is a key piece behind the motivations for my project. Further, the association between NGF and BDNF can further be correlated to our study on TNF α. Our collective efforts to understand cytokine and neurotrophin signalng may justify targeting this signaling triad pathway in the treatment of MS.

While, the results of this study indirectly support the need to carry out my research, my participation has further advanced my learning and development of technical skills. Specifically, my association with this study has allowed me to develop confident skills in gene analysis using qRT-PCR.

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CHAPTER 4: SPINAL CORD BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) RESPONSIVE CELLS IN AN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) MODEL OF MULTIPLE SCLEROSIS (MS): IMPLICATIONS IN MYELIN REPAIR

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STATEMENT OF CONTRIBUTION

For this manuscript, I was responsible for performing qRT-PCR for the TrkB gene in SC samples, including isolating the 146 bp TrkB gene from an agarose gel, and running the standards. I induced, monitored, and perfused the animals used in preparation for transmission electron microscopy (TEM). Additionally, I was heavily involved in the revisions of the manuscript. My involvement in this project was instrumental in assisting me to develop independent proficiency in the various analytical techniques described in detail in the following manuscript.

CHAPTER 4

4.1. ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease that destroys central nervous system (CNS) myelin. Although, the exact pathophysiology of MS is unknown, it is associated with CNS infiltration of T-cells and monocytes, which subsequently activate phagocytic cells that directly damage myelin. Brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase receptor (TrkB), have recognized roles in myelin structure formation, maintenance, and repair. We used an experimental autoimmune encephalomyelitis (EAE) model of MS to determine changes in TrkB expression that may contribute to neurological recovery and myelin repair following an early inflammatory immune-mediated attack on CNS myelin. Spinal cord (SC) TrkB gene and protein expression were analyzed at various time intervals post-EAE induction. Analysis of gene and protein expression was conducted in animals with EAE relative to active controls (AC) and naïve controls (NC). We showed significant increases in TrkB protein in the SC of EAE rats 12 days post-induction relative to controls. This elevated TrkB expression correlated with the onset of neurological recovery days 12 to 15 post-EAE induction. Furthermore, immunohistochemistry (IHC) analysis revealed up-regulated expression of TrkB in several SC cell types including a specific subset of BDNF responsive neuronal cells. Finally, transmission electron microscopy (TEM) showed the ultrastructural integrity of myelin is already compromised during the early, inflammatory stage of EAE prior to widespread demyelination. Therefore, the molecular signaling of SC BDNF via TrkB represents a key therapeutic target whose manipulation

could facilitate myelin repair and neurological recovery following an MS-induced myelin attack.

Keywords: EAE; TrkB; spinal cord; multiple sclerosis.

4.2. INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) [1]. There are essentially two phases of disease, an inflammatory phase involving the infiltration of active T-cells and monocytes into the CNS, followed by a second phase of disease involving immune mediated cell death of oligodendrocytes (OGs) [1]. Due to the lack of human tissue samples, MS research has largely relied on various animal models [2, 3] to explore the intricate cellular signaling that regulates myelin structure, function and repair. Animal models using myelin basic protein (MBP) as the antigen, characteristically activate lymphocytes that mediate an inflammatory response without demyelination [4]. One of the characteristic pathological features of MS is the identification of segments of demyelinated axons with subsequent death of OGs [5-8]. Oligodendrocyte progenitors (OPs) attempt to proliferate and become mobilized for subsequent recruitment to the sites of injury in order to re-myelinate denuded axons [7, 9, 10]. However, they are unable to completely repair the damaged myelin. The cellular mechanisms that govern this "insufficiency" are currently unknown. Although several studies have shown that OPs exist in the CNS [11-13], their inability to appropriately migrate, proliferate and differentiate at the site of SC injury results in partial or incomplete myelination [7, 14, 15]. Previous studies have tried to target the effects of growth factors on OPs to optimize this process but these efforts have resulted in only marginal success [13, 16, 17]. Thus, the mechanism for re-myelination of SC white matter is still unknown. Henceforth, the current research is focusing on examining the

time dependent expression changes of TrkB during the early inflammatory phase of EAE in the SC as a possible mechanism of cellular responsiveness to local BDNF release.

4.2.1. REGULATION OF MYELIN FORMATION VIA BDNF

The neurotrophin, BDNF, has been suggested as a critical factor involved in re-myelination and/or the structural repair of myelin [18, 19]. However, the specific molecular mechanism(s) linking BDNF activity to SC myelin repair are not well defined. Furthermore, little is known about the exact molecular mechanisms by which BDNF/TrkB regulates the myelin-producing cells and the structural protein composition of myelin. Irrespective, part of the beneficial effects of BDNF result from its direct ability to induce OP proliferation [20], migration and differentiation at the site(s) of injury [21], which are essential steps in myelin formation [22, 23]. In addition to regulating OG lineage cells, BDNF also regulates other cell types (including astrocytes and neurons) that are also critical for myelination [24]. Furthermore, BDNF is responsible for regulating the expression of the main structural proteins of myelin such as MBP [25], myelin associated glycoprotein (MAG), and proteolipid protein (PLP) in mature OGs [22, 23, 26]. In addition, we have also published research that provides key evidence in support of BDNF and myelin repair. Our recent study demonstrated that following EAE induction, BDNF was up-regulated in the DRG and delivered to the SC [27]. Specifically, small to medium-sized sensory neurons housed within the DRG were identified as the cellular source of BDNF being delivered to the SC [27]. As a result, DRG-derived BDNF

is now recognized as an essential requirement for SC myelin repair and subsequent neurological recovery following an immune system mediated attack.

4.2.2. BDNF MOLECULAR SIGNALING

BDNF is synthesized and secreted as a precursor protein, called *proBDNF (34 kDa)*, which can be proteolytically-cleaved to produce a *mature form (13 kDa)* [28-30]. In general, BDNF signaling is regulated *via* a receptor complex that encompasses two different receptor classes: the tropomyosin-related kinase (Trk) receptor B (TrkB) [31, 32] and the $p75^{NTR}$ receptor, a member of the tumor necrosis factor (TNF) receptor superfamily [32, 33]. Previous studies have shown that SC OGs do not express TrkB, but do express $p75^{NTR}$ [34, 35]. The mature, 13kDa form of BDNF only signals *via* the TrkB receptor [31], whereas, proBDNF (34 kDa) interacts with the p75 neurotrophin receptor (NTR) [36] to mediate biological actions distinct from those of TrkB [32, 37, 38]. Although there are no published studies addressing TrkB expression in SC OGs, there are studies showing that proBDNF promotes myelination *via* $p75^{NTR}$-mediated activity independent of TrkB [39-41]. Specifically, inhibition of the $p75^{NTR}$ receptor activity prevented the expression of one of the key structural proteins of myelin called MAG [39]. Thus, proBDNF is critical for the structural integrity of the intact myelin sheath through TrkB-independent pathways. However, despite the apparent beneficial roles of BDNF/TrkB in myelin repair, its exact mechanism is still unknown [27].

We hypothesized that SC BDNF responsive cells contribute to myelin repair and neurological recovery following an immune system mediated attack on CNS myelin. As a result, an MBP-EAE animal model of MS was specifically used to identify the BDNF responsive cells during the early inflammatory phase of the disease process prior to demyelination.

4.3. MATERIALS AND METHODS

4.3.1. EAE MODEL & NEUROLOGICAL TESTING

Experimental autoimmune encephalomyelitis (EAE) was induced using MBP, in adolescent female Lewis rats (Charles River, Montreal, QC) as previously described [27]. A total of 66 adolescent female *Lewis* rats were divided into 3 experimental groups: *naïve control (NC), active control (AC)* and *experimental autoimmune encephalomyelitis (EAE).* NC animals *(n=6)* did not receive any injections. AC animals *(n=30)* received 2 intraperitoneal injections *(I.P.)* of pertussis toxin (PT; List Biological Laboratories CN: 180) (0.3 µg in 200 µl of phosphate buffered saline (PBS; Sigma: P-5368) at the identified time point′s day 0 & 2. In addition, these animals also received 2 x 50 µl s.c bilateral injections of Freund′s adjuvant (FA; (Sigma, CN: F-5506) + 500 µg of Mycobacterium Tuberculosis H37Ra (MT; Difco Laboratories, CN: 3114) + saline into the area above the base of the tail at day 0. *EAE animals (n=30)* received the same PT regimen administered to *AC* plus *full inoculation* with FA + 500 µg MT + 100 µg Guinea

pig myelin basic protein (MBP; Cedarlane, CN: GP68-84) given as 2 x *50 µl s.c* injections into the area above base of the tail. Animals in the AC and EAE groups were sacrificed for comparative protein and gene analysis at day 3, 6, 9, 12, and 15. Acronym identifier such as EAE3 refers to EAE animals - 3 days after inoculation vs AC3 refers to AC animals 3 days after inoculation and so forth. All animal experiments were conducted in accordance with the University of Manitoba Animal Users and Protocol Management Review Committee protocols, which comply with the Canadian Council on Animal Care guidelines. Our established experience with this model [42] indicates that EAE animals, begin to develop mild clinical symptoms by day 9 (tail weakness or paralysis) [43]. By day 12 to 13 all animals experience a full range of neurological deficits such as tail and forelimb weakness, loss of bladder control and hind-limb paralysis. Based on our previous experience, with this model, the EAE rats enter into remission and regain motor function by 15 days post EAE induction. *Female* rats were specifically chosen because they are more predisposed to be affected than males [44]. Neurological disability is scored according to the criteria detailed previously [45]. Neurological disability is scored according to the following criteria: *Tail*: 0 = normal; 1 = partially paralyzed, weakness; 2 = completely paralyzed, limp; *Bladder*: 0 = normal, 1 = incontinence; *Right hind limb*: 0 = normal, 1 = weakness, 2 = dragging with partial paralysis, 3 = complete paralysis; Left *hind limb*: 0 = normal, 1 = weakness, 2 = dragging with partial paralysis, 3 = complete paralysis; *Right forelimb*: 0 = normal, 1 = weakness, 2 = dragging, not able to support weight, 3 = complete paralysis; *Left forelimb*: 0 = normal, 1 = weakness, 2 = dragging, not able to support weight, 3 = complete paralysis. SC tissue will be collected at days 3,

6, 9, 12 and 15. Whole DNA/RNA and protein was collected using commercially available kits as described in our previous publications [27, 46, 47]. Changes in TrkB gene and protein expression levels will be assessed by standard in house protocols for immunohistochemistry (IHC), real time RT-PCR, and western blotting (WB) [27, 45-48].

4.3.2. REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was isolated from SC tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The mRNA was transcribed to cDNA and quantitative real time RT-PCR was conducted for measuring target gene as previously described [27], using a Light-Cycler-DNA master SYBR green-1 kit following the manufacturer's protocols (Bio-Rad, Hercules, CA, USA). TrkB primers were: forward: 5'-acgtcaccaatcacacggagtacc-3'; reverse; 5'-ctggcagagtcatcgtcgttgc-3', annealing temperature 62°C. PCR product was calculated to a length of 430bp, as was confirmed on an ethidium bromide agarose gel. GADPH primers were: forward: 5'-aagaaggtggtgaagcaggcg-3'; reverse; 5' – agacaacctggtcctcagtgtagc-3'.

4.3.3. WESTERN BLOT (WB)

Protein concentration for each sample was assessed using the Bradford protein assay. For each sample, 30 µg total protein was analyzed by WB as previously described [27]. Anti-

TrkB antibody (1:500, R&D system Minneapolis, MN, USA) was used to detect BDNF protein. Following incubation with anti-rabbit secondary antibody (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA), the enhanced chemiluminescence signal was detected by Multi Image ®II FluorChem®FC2 (Alpha Innotech, Santa Clara, CA, USA). Signals were normalized to GAPDH (1:1000; R&D Systems). Densitometry readings were performed using a FluorChem 8900 scanner (Alpha Innotech, San Leandro, CA, USA) with Alpha Ease FC software, and relative density ratio was calculated for the individual TrkB band density for each sample, vs. GAPDH levels. Three different cell preps were assayed by western blot and the mean densities for each band calculated, and their mean value was used [49].

4.3.4. IMMUNOHISTOCHEMISTRY (IHC) ANALYSIS

For IHC analysis of protein expression, animals were perfusion fixed with 4% paraformaldehyde as previously described [27]. Spinal columns were dissected free of overlying muscle and connective tissue, and decalcified according to previously described protocols [48]. Qualitative IHC analysis of 10 µm cryostat sections were conducted according to previously described methods [27]. Double-labeled immunofluorescence using antibodies against MBP (1:50, Santa Cruz), GFAP (1:100, Santa Cruz), CD68 (ED1) (1:100, Santa Cruz), PDGFR-α (1:50, Santa Cruz), CD4 (1:100, Santa Cruz), and neuron specific β-III Tubulin (1:50, R&D) were conducted in conjunction with the polyclonal antibody TrkB (1:100, R&D system Minneapolis, MN,

USA). Imaging was conducted using an Olympus BX51 configured with FV5000 confocal laser scanning capability. Acquired images were captured in Fluoview Version 4.3. Image sizing, black background balancing and final collation for publication were performed using Adobe Creative Suite 2 v9.0.2 (Adobe Systems Inc., San Jose, CA, USA). Cell size analysis was performed using Image Pro Express software (Media Cybernetics, Bethesda, MD, USA). No image manipulations were performed other than those described. Primary antibody and secondary antibody omission studies were also conducted on the samples *(data not shown)*

4.3.5. SIZE ANALYSIS OF SC DORSAL HORN NERVE FIBERS

The diameters of cross-sectional axons in SC tissue expressing TrkB were measured. TrkB positive (TrkB+) neurons were identified and measured in each of the 10 µm thick SC sections obtained from each experimental group (NC, AC, EAE). Imaging was performed using an Olympus IX81 scanning laser confocal microscope. Images were captured in Fluoview FV500 software. Image processing and nerve fiber diameters were measured (image of 100X magnification were used) using the Image Pro Express V6.0 (Media Cybernetics) software. To measure the nerve fiber diameters, a line was drawn from one edge of the axon to the opposite end to obtain a cross-sectional width of the nerve fibers. Measurements are expressed in microns. Statistical analysis of cell sizes were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The data were analyzed using one way

analysis of variance (ANOVA) with Bartlett's test (for equal variances) and Dunnett's Multiple Comparison Test. Significance was set at $P < 0.05$. Significant differences between the means of different observational groups were determined.

4.3.6. TRANSMISSION ELECTRON MICROSCOPY

SC ultrastructure was observed with TEM for changes in myelin protein structure and integrity. Animals from each experimental group (n =1) at day 12 post inoculation were perfused via left ventricle with a pre-fix solution containing 1I.U./ml heparin and 1% sodium nitrate in 0.9 % sodium chloride followed by a fixative solution of 4% of paraformaldehyde in Sorensen's buffer (pH 7.3). Whole SCs were extracted and dissected into 1 mm by 1 mm sections and incubated by immersion with the same fixative solution for 3 hours, and then the solution was replaced with 5% sucrose in 0.1 M Sorensen's buffer for 1 hour. Tissue sections were post-fixed using 1% of osmium tetroxide. The samples were dehydrated in ascendant alcohol and subsequently embedded in epon812 resin (E.M.S., Embed-812 embedding kit, 14120). Thin sections were stained with uranyl acetate and counterstained with lead citrate. SC ultrastructure was observed and micro photographed with a transmission electron microscope Philips CM10, at 60 kV, on ultra-thin sections (100 nm on 200 mesh grids) at the Histomorphology & Ultrastructural Imaging Unit of the University of Manitoba.

4.3.7. STATISTICAL ANALYSIS

Statistics was performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Results are expressed as mean ± SEM. The data were analyzed by one-way ANOVA with Tukey's Multiple Comparison post hoc test. Student's *t*-test was used to assess significance of differences between the groups. Normality and homogeneity of error variance of dependent variable was tested by using Kolmogorov-Smirnov and Levene's test. A value of $P < 0.05$ was considered significant.

4.4. RESULTS

4.4. 1. NEUROLOGICAL DISABILITY

All animals in the EAE groups were assessed for neurological disability according to previously described protocols [27, 47, 50]. None of the animals displayed neurological deficits prior to day EAE6 as evident by a score of zero. However, all EAE animals started to display signs of neurological disability by EAE9 representing the early inflammatory stage of disease induction (0.57 ± 0.45) (**Figure 1**). Mean peak neurological disability score (6.42 ± 5.35) was achieved by EAE12 which subsided by EAE15 (1.5 ± 1.41) as the animals entered the remission phase of disease. This pattern of neurological disability is well characterized for this animal model, and it has been

consistent with our previous publication, give us the same graph published by [47] published in BioMed Research International. In contrast, NC and AC animals did not display neurological symptoms *(data not shown).*

4.4. 2. EXPRESSION OF TrkB PROTEIN IN THE EAE SC

WB was used to determine the comparative time dependent TrkB protein expression amongst the 3 experimental groups (NC, AC and EAE). WB analysis revealed a significant increase in TrkB expression in EAE9 and EAE12 groups compared to NC and AC9 and AC12 groups (**Figure 2A**). Densitometry was conducted on the blots, and TrkB protein levels were assessed as a ratio of GAPDH levels. NC animals show TrkB protein expression at 0.235 ± 0.02. AC9 and AC12 animals show increased protein expression of TrkB relative to NCs (AC9 = 0.576 ± 0.12; AC12 = 0.807 ± 0.15). In comparison, EAE9 and EAE12 animals show a significant increase of TrkB expression in the SC over NCs and AC9 and AC12 (EAE9 = 2.124 ± 0.24, $P < 0.05$ and EAE12 = 2.24 ± 0.26, $P < 0.05$) (**Figure 2B**).

4.4. 3. EXPRESSION OF TrkB mRNA IN THE EAE SC

Real time RT-PCR was used to assess TrkB mRNA expression from all NC, AC and EAE groups. The data was normalized with GAPDH expression from each sample. Statistical analysis shows no significant difference in TrkB mRNA expression between

EAE and AC groups (**Figure 2C**). However, EAE12 and EAE 15 animals did show a significant increase in mRNA expression over NC group ($P < 0.01$ and $P < 0.05$ respectively) (*$P < 0.05$; ** $P < 0.01$; ANOVA followed by Tukey's posthoc test).

4.4.4. IHC ANALYSIS OF BDNF RESPONSIVE CELLS IN THE SC GLIAL CELLS

Double-labeled IHC staining was conducted to identify various types of BDNF responsive cells ***(TrkB expressing cells)***. A variety of cellular markers including: CD68 (macrophages, GFAP (astrocytes), PDGFRα (oligodendrocyte precursors) and MBP (mature oligodendrocytes) were used to determine the expression of TrkB on glial cells. Our results show that TrkB was not co-localized with any of the other glial cell markers for which we have examined in the dorsal horn of the EAE SC *(Data not shown).*

4.4.5. IHC ANALYSIS OF BDNF RESPONSIVE CELLS IN THE SC IMMUNE CELLS

IHC analysis identified TrkB protein co-localization with CD4+ T-cells (**Figure 3A**) and Th17 cells in EAE SC (**Figure 3B**). These results confirm that immune cells express the TrkB receptor in EAE SC.

4.4.6. IHC ANALYSIS OF BDNF RESPONSIVE CELLS IN THE SC NEURONAL CELLS

IHC analysis of EAE SC identified TrkB co-localization with the neuronal marker β-III-tubulin (**Figure 4).** Interestingly, IHC analysis also showed enhanced TrkB+ cells being localized specifically to the posterior marginalis and the nucleus proprius layers (**Figure 5A**) of the dorsal horn. IHC analysis suggests that the specific subsets of BDNF responsive neurons are likely to be Aδ fibers. The reason being is that Aδ fibers originate in the dorsal root ganglia and synapse in the posterior marginalis.

4.4.7. MEASUREMENT OF NERVE FIBER DIAMETER IN THE DORSAL HORN OF THE SC

Dorsal horn nerve fibers expressing TrkB showed a statistically significant ($P < 0.0001$) increase in size in the EAE12 group compared to AC animals at the same stage of the disease progression. NC SC nerve fibers had a mean diameter of 1.12 ± 0.34 µm, compared to AC animals with a mean diameter of 1.27 ± 0.33 µm. EAE animals have a mean nerve fiber diameter of 2.01 ± 0.58 µm which is highly significantly different from the AC nerve fiber diameters (***$P < 0.0001$) (**Figure 5B**). These results suggest that the TrkB+ neuronal cell population switches from C fibers to Aδ fibers involvement following immune system activation. These findings correlate with the IHC findings described above, shown in **Figure 5A**.

4.4.8. MYELIN PATHOLOGY IN THE SC OBSERVED IN TEM

Since animals induced to a state of EAE exhibit peak neurological disability 12 days post-EAE induction, we aimed to assess the effect of EAE disease state on the structure and integrity of the myelin sheath. We believe changes in myelin structure and integrity contribute to the development of neurological disability seen in animals with EAE. We analyzed lumbar SC ultrastructure using TEM to assess the comparative assessment of TEM images for NC, AC12, and EAE12 animals reveal significant myelin pathology during EAE (**Figure 6 I-V**). TEM images of the SCs of NC and AC12 animals reveal normal myelin ensheathment of various sized axons with no differences between the two groups (**Figure 6 I, II, III**). However, SC of EAE12 revealed the myelin sheath surrounding the axons of small to medium diameter neurons are markedly thinner than in both healthy and non-disease controls (**Figure 6 IV, V**). In addition, there is pronounced absence of myelin ensheathment in large diameter axons, as well as, the presence of necrotic cells. These changes in myelin integrity correlated with peak neurological disability (**Figure 1**).

4.5. DISCUSSION

Although the exact mechanisms underlying MS remain unknown, several therapeutic candidate molecules have been suggested to have a significant beneficial role in repairing damaged myelin and reversing the neurological disabilities associated with this white

matter disorder [45, 51]. Specifically, recent studies have identified BDNF as a critical regulator of myelin repair following immune system mediated destruction of CNS myelin [27, 39, 52, 53]. However, there is little understanding of the exact role of BDNF in MS. We hypothesized that the TrkB receptor for BDNF represents a critical component essential to the beneficial molecular signaling of BDNF that is involved in myelin repair following EAE induced myelin damage. Therefore, we designed a study focused on assessing the time dependent expression changes of the high-affinity receptor TrkB in the SC following EAE induction.

Our data demonstrates significant increases in the protein expression for TrkB in the SC of EAE compared to NC animals and AC animals at the same time points following EAE induction. Interestingly, based on the SC distribution of the TrkB+ cells within the dorsal horn (**Figure 5A**), we show that these BDNF responsive cells correspond to the anatomical location and nerve fiber diameter of those neurons involved in the transmission of pain. Based on our earlier published EAE studies [27], we demonstrated that BDNF is up regulated at day 12 in the dorsal horn of the SC following EAE induction [27]. Interestingly BDNF signaling via TrkB in the dorsal horn of the SC is known to contribute to neuropathic pain (NPP) by activation of microglia [54]**.** NPP is a chronic syndrome commonly suffered by patients with MS [55]. It is proposed NPP and the early induction of MS share molecular mechanisms [56]. As such, up to 50% of MS patients present with NPP prior to their diagnosis or in the earliest stages of disease progression [57-59], which suggests the development of NPP in patients with pre-

disposing risk factors for MS may serve as a pre-diagnostic indicator for MS. Therefore, the up regulation of BDNF [27] and increase in cellular responsiveness (via TrkB) to local BDNF release in the dorsal horn SC may contribute to the development of NPP. Thus, an elevation in BDNF and TrkB may likewise serve as early indicators for the development of MS.

In this study we showed a significant up-regulation in TrkB protein expression (EAE 9 - 15) throughout the SC which suggests increased cellular responsiveness to BDNF via TrkB thereby contributing to neurological recovery. This is consistent with the findings of Song et al., which showed that reduced TrkB expression increased EAE disease severity [60]. Interestingly, our IHC data showed the absence of TrkB expression in SC astrocytes during EAE. However, recent SC injury studies indicate that the TrkB receptor on astrocytes sequester the incoming BDNF protein in injured SC [61, 62], thereby preventing BDNF from exerting its beneficial effects on re-myelination and/or myelin repair. Our data in this regard, suggests that BDNF sequestering may not be possible due to the absence of astrocyte TrkB expression. Thus, this would allow DRG-derived BDNF to promote neurological recovery/remission and exert its effects on myelin repair following an EAE induced insult to CNS myelin. BDNF signaling via TrkB has been shown to be protective, reducing disease severity during the early mild phase of EAE [60]. Our results using an MBP inflammatory model of EAE also support these findings. Interestingly, this is not the case during severe disease [63]. In fact, it has been demonstrated that an increase in TrkB expression in astrocytes in MS and EAE lesions

facilitates nitric oxide production and neurodegeneration that inhibit re-myelination [64-67]. As such, researchers must be aware of the differential effects that correspond to the differential progression stages of MS. Hence, these later findngs suggest that in the later stages of disease progression TrkB signaling may modulate disease outcome and severity. Thus differential TrkB signaling may lead to different biological outcomes that are dependent on time throughout disease course. Accordingly, it is not surprising then that during an early inflammatory pre-demyelinating model of EAE we noted an absence of astrocyte TrkB expression. This is likely because the duration of the study was too short to demonstrate long term disease progression. Henceforth, the lack of TrkB containing astrocytes during early EAE induction may help account for recovery from neurological disability by EAE15. As a result, our study identifies that the early inflammatory stages following EAE may represent the most optimal time for targeted treatment strategies to reverse the downstream effects of myelin damage that are known to occur as a function of time. However, additional studies are required to confirm this.

As a result, there appears to be a narrow window of time in the early stages of MS by which BDNF can beneficially signal through TrkB to promote myelin repair. As the disease progresses, there appears to be a continued elevation of TrkB containing astrocytes. These TrkB+ astrocytes eventually take over and prevent complete myelin repair at the lesion site by sequestering BDNF. As a result, patients in the later stages of disease progression are left with a permanent neurological disability for which they can’t recover. Based on the current knowledge of MS, patients with relapsing remitting MS

(RRMS) eventually develop secondary progressive MS (SPMS). SPMS is the clinically recognized progressive phase of the disease where patients experience a continued decline of neurological function without any periods of remission/myelin repair. The TrkB findings presented from our research in the early inflammatory stages of EAE taken together with the findings of other EAE researchers involving the later stages of the disease, confirm the importance of BDNF and TrkB in myelin repair. Our research also suggests the importance of astrocytes in preventing myelin repair in the later stages of MS.

Interestingly, in our study we did not see TrkB co-localized with oligodendrocyte immunoreactivity. This contradicts previously published studies, which have shown SC OGs express TrkB [68]. The difference between our study and the previous studies may be as a result of the use of different antibodies to identify cells of the oligodendrocyte lineage, or the type of animal model being studied.

Furthermore, we did not identify TrkB+ cells in NC SC grey matter, which correlates with one previous study [68]. However, it is possible that TrkB expression in OGs does not occur in the early stages of the disease progression, further studies of the later secondary de-myelinating phase of the disease may reveal a role for BDNF in the direct regulation of re-myelinating events. Alternatively, BDNF may regulate OGs via pro-BDNF via $p75^{NTR}$ interactions on OGs. ProBDNF is known to promote myelination *via* p75NTR-mediated activity independent of TrkB [39]. BDNF binds to two neurotrophin

receptors, p75^{NTR} and TrkB [37]. Pro-BDNF preferentially binds to p75^{NTR} [69], and the mature form of BDNF binds with high affinity to the TrkB receptor [70]. Studies show that OGs express p75^{NTR} , expression of which is down regulated after axonal contact is made [35]. Thus, further studies are required to identify the differential expression of p75^{NTR} and TrkB in OG lineage cells.

Several studies have shown that CD4^{+} cells are an important source of BDNF that contributes to immune mediated neuroprotection [71]. Our results show that cells of immune origin specifically CD4^{+} Th cells and Th17 cells, also express TrkB and are therefore responsive to BDNF signaling. BDNF appears to regulate the differentiation of thymocytes to their CD4^{+} or CD8^{+} mature status [72]. Thus it is plausible that in the early stages of EAE disease progression (as assessed in our study), the TrkB+ CD4^{+} cells we identified are recently differentiated Th cells. These cells may lose their BDNF responsiveness as they mature. Th17 cells have recently been identified as playing a critical role in the MS disease process [73]. However, there have been no studies investigating the role of BDNF in the regulation of Th17 cells. It is possible that their differentiation is also regulated by BDNF in the early stages of maturation. It is also possible that BDNF acts to protect the Th17 cells from activation-induced apoptosis [74]. Further studies are required to confirm a role for BDNF in regulating Th17 cells. Inflammatory responses can provide regenerative and protective effects during CNS damage [75]. Thus the TrkB+ T-cells could play an important role in MS immunopathogenesis by modulating auto-reactive T-cells.

Our study also shows that TrkB immunoreactivity is localized specifically to the axons of SC in the EAE12 experimental group. This localization of TrkB in the SC axons may represent the earliest stages by which any axonal transport of BDNF from the DRG may be sequestered thereby preventing it from reaching its target site at the MS lesion. Interestingly, qRT-PCR data show that there is no significant change in TrkB mRNA expression between EAE and AC groups. These results suggest that increased SC TrkB protein expression may be as a result of increased translation in the axons. Our previous study showed that the predominant source of BDNF protein expression in the DRG is small diameter (1-5 μm) Aδ sensory nerve fibers [27]. Consistently, our current study identifies Aδ fibers as the predominant TrkB+ (BDNF responsive) cells in the dorsal horn. Additionally, while large diameter axons are absent of myelin, small to medium sized axons exhibit only a marked reduction of myelin ensheathment based on TEM analysis. Although, a marked thinning of myelin coating in small to medium diameter neurons may compromise the structural integrity of Aδ fibers, BDNF transport mechanisms may likely still be functional during EAE [27]. Further, our results suggest that the TrkB+ neuronal cell population switches from C fibers to Aδ fibers following immune system activation, which may serve to increase BDNF transport from the DRG to SC. As a result, the increase expression of TrkB in Aδ fibers and the presence of thinly myelinated small to medium axons support our hypothesis that the transport of DRG-derived BDNF into the SC via the DRG-SC connective pathway is critical for neurological recovery and myelin repair.

Sensory neurons are classified according to conduction velocity, which easily translates to axonal diameter. The smallest fibers, C and Aδ fibers, are pain and temperature receptors, and synapse in different layers of the dorsal horn. The three main layers of the dorsal horn are the posterior marginalis, the substantia gelatinosa, and the nucleus proprius. Aδ pain fibers enter the posterior marginalis and the nucleus proprius, and synapse with sensory afferent neurons that carry pain signals to the thalamus via the spinothalamic tract. The spinothalamic system is one of the major pain-transmission pathways [76]. The C fibers enter the substantia gelatinosa and synapse on interneurons, which do not project out of the immediate area. Thus our data showing that EAE SC has a higher proportion of Aδ fibers suggests that the mode of BDNF induced pain transmission is altered during the course of the disease. In addition, the expression of TrkB in the nucleus proprius shows that the Aδ fibers are signaling directly with sensory afferents associated with the spinothalamic tract. This finding correlates with our studies showing that EAE animals are more sensitive to cold than active control animals at the same phase of the disease [50].

4.6. CONCLUSION

Our study provides evidence of BDNF responsive cells in the SC in an animal model of MS, which support the role of TrkB in modulating acute inflammatory processes that contribute to myelin damage and associated neurological disability. We observed an up-regulation of TrkB in SC at day 12 post-EAE induction, while Zhu et al. in 2012 showed

an up-regulation of BDNF in SC at the same time point. Both these findings correspond to the inflammatory peak of the acute phase of EAE. Further, we revealed TrkB expression is up-regulated in several SC cell types including Aδ nerve fibres but absent in astrocytes. Finally, we showed myelin ensheathment is compromised in various size axons in an EAE model normally associated with little to no demyelination.

Other studies show BDNF is protective during the early mild phase of EAE but not during severe disease. This may be explained by astrocyte sequestering of BDNF via TrkB which prevents BDNF from exerting its beneficial effects. Since our study does not show TrkB expression in astrocytes, we suggest the TrkB receptor may be a disease modulator dependent on disease time course and cell type. Therefore, this suggests a narrow window may exist during the early inflammatory phase of EAE when a precise balance between ligand (BDNF) and receptor (TrkB) is achieved to promote neurological recovery. Therefore, this may provide insights into the optimal time for early treatment in MS patients. Further studies are also required to study the expression of $p75^{NTR}$ receptor in EAE SC to determine the effect of TrkB independent BDNF activity.

4.8. FIGURES

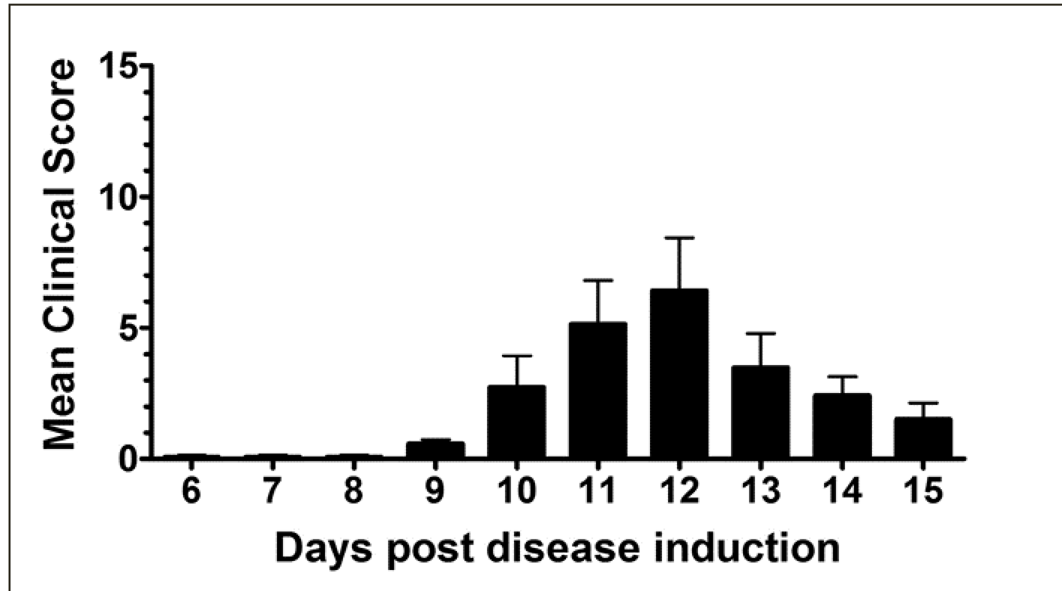

Figure 1: Neurological Disability Clinical Score for EAE animals

This figure illustrates clinical scores in the EAE animals, at different times in the disease progression. Global neurological disability score obtained from assessment of all 6 specific clinical domains outlined in Materilas and Methods (EAE model & Neurological Testing) Disability scores range from a mean clinical disability score of 0 (no disability) to 15 (maximal disability). Results indicate EAE animals developed signs of neurological disability at EAE 6 that peaked at EAE12 which subsided by EAE14 similarly to [47].

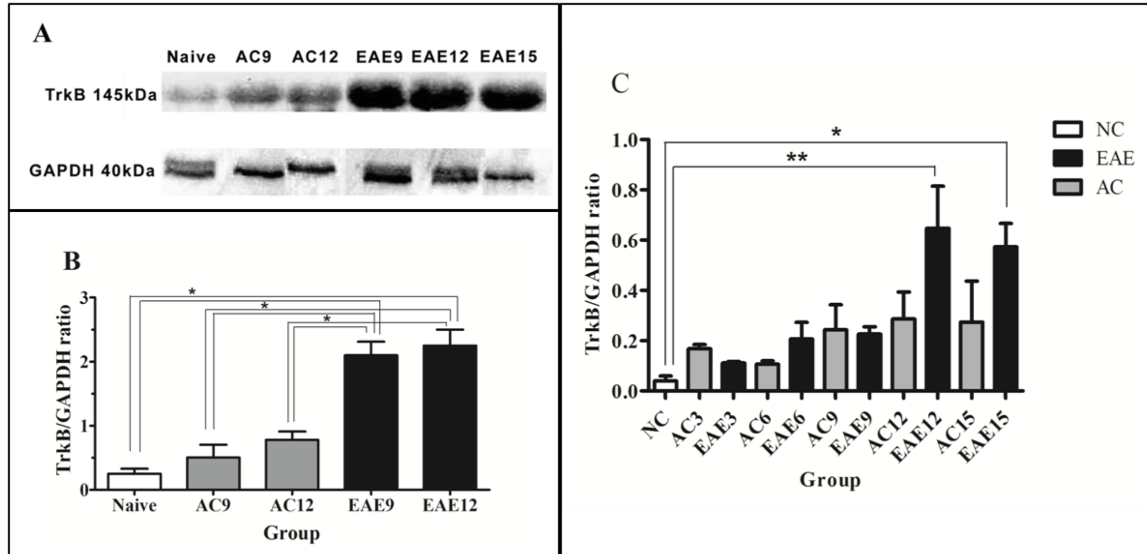

Figure 2 A – C: TrkB protein and gene expression in the SC. **(A)** WB analysis of TrkB protein expression in the SC illustrates that full length TrkB (145kDa) protein is detected in naïve, active control and EAE groups at the different time points during disease progression. GAPDH protein was used as a loading control, and is detected at 40 kDa. (EAE9 = EAE animals 9 days post-inoculation; AC9 = 9 days post-inoculation; Naïve = NC animals). **(B)** Densitometric analysis of WB for TrkB in SC shows increased TrkB protein expression in the SC of EAE animals (black bars) compared to NC (white bars) & AC (grey bars) SC, at different times in the disease progression. NC animals (white bars) show TrkB protein expression at 0.235 ± 0.02. AC9 & 12 animals [AC9 = 0.576 ± 0.12 and AC12 = 0.807 ± 0.15 (grey bars)] shown increased protein expression of TrkB compared to NCs. However, analysis of EAE animals (EAE9 = 2.124 ± 0.24, $P < 0.05$ and EAE12 = 2.24 ± 0.26, $P < 0.05$) show a significant increase of TrkB expression in the SC when comparatively assessed to NC (0.235 ± 0.02) and AC 9 &12 (AC9 = 0.576 ± 0.12 and AC12 = 0.807 ± 0.15). **(C)** This figure illustrates TrkB mRNA

gene expression in SC at different times in the disease progression. Comparative Real time RT-PCR of TrkB expression in SC shows no significant difference in mRNA expression between the AC and EAE animal groups. However, EAE animals did show a significant increase in mRNA expression over NC group [NC = 0.0400 ± 0.0200] at days 12 and day 15 [EAE12 = 0.6467 ± 0.1683 EAE12 = 0.5733 ± 0.0933] (*$P < 0.05$; ** $P < 0.01$; ANOVA followed by Tukey's posthoc test). Errors bar represent standard error of mean (SEM).

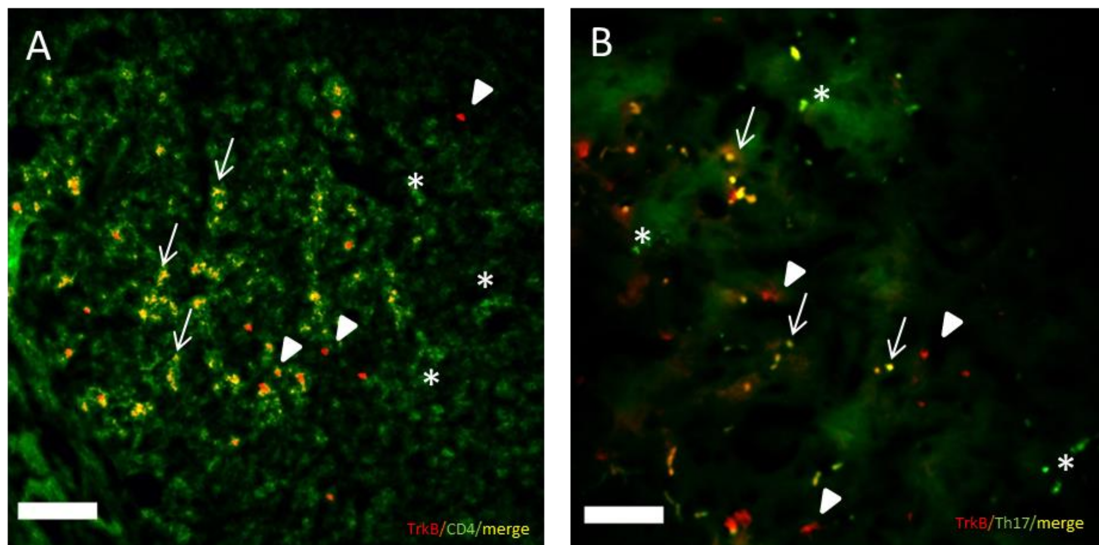

Figure 3 A – B: TrkB expression by immune cells in the EAE SC. **(A)** TrkB and CD4 double labeling in EAE SC: This figure illustrates that IHC results (TrkB labeling red and CD4+ T cell labeling green) show TrkB is co-localized with CD4+ T cells (yellow). Images were taken at a total magnification of 100X from EAE 12 group. (Arrowheads depict TrkB labeling, * depict CD4+T cell labeling and arrows depict cells co-labeled for CD4+ T cells and TrkB). Bar = 20µm. **(B)** TrkB and Th17 double labeling in EAE SC: This figure illustrates that IHC results (TrkB labeling red and Th17 labeling

green) show TrkB is co-localized with Th17 cells (yellow). Images were taken at a total magnification of 100X from EAE 12 group. (Arrowheads depict TrkB labeling,* depict Th17 labeling and Arrows depict co-labeling between TrkB and Th17 cells). Bar = 20μm.

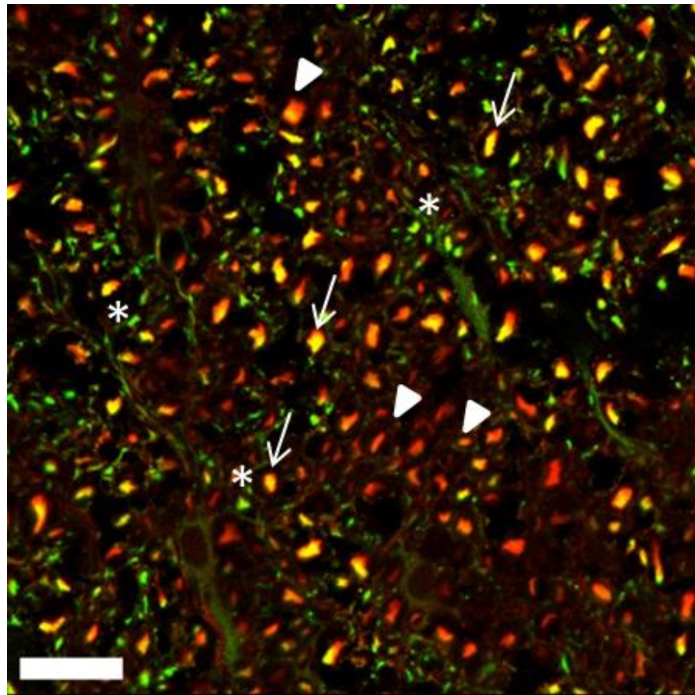

Figure 4: TrkB expression by neurons in the EAE SC. This figure illustrates that IHC results (TrkB labeling red (arrowheads) and β-III Tubulin labeling green (*) show TrkB is co-localized with neuronal axons (yellow- arrows). Images were taken at a total magnification of 100X from EAE 12 group. Bar = 20μm.

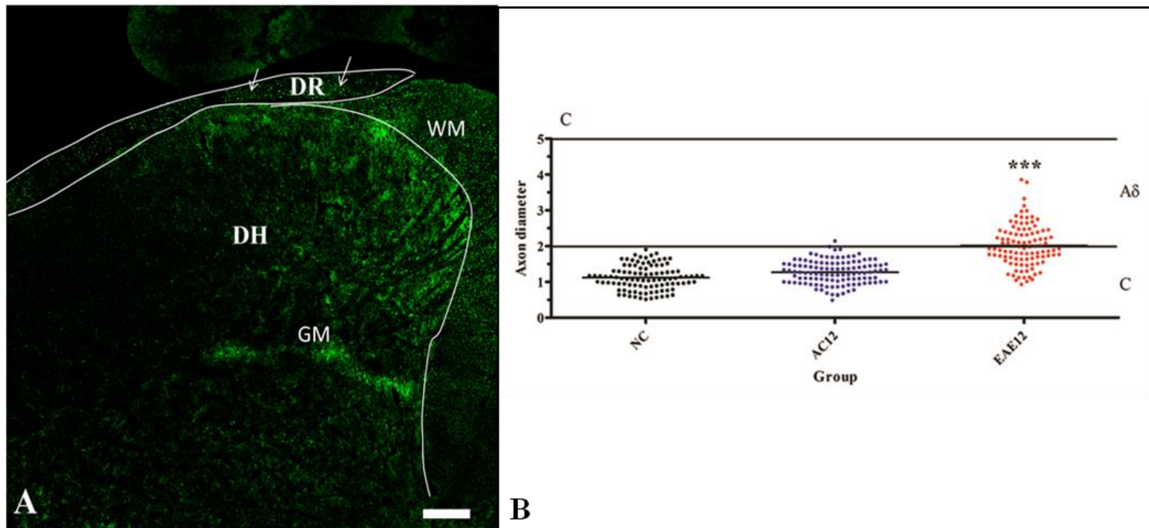

Figure 5A – B: IHC analysis of TrkB positive cells in the dorsal horn of the SC. (A) IHC staining of TrkB localization in the SC of EAE 12 show that BDNF responsive cells are located in the posterior marginalis at the dorsal root entry zone, and also in the nucleus proprius. Such cells correspond with Aδ fibers, which connect to the spinothalamic tract. IHC of TrkB in the SC of EAE12**,** shows TrkB (green spots) in the dorsal root **(DR:** white arrows indicate the dorsal root entry point) and grey matter **(GM)** of the SC. **WM** = white matter. Total magnification 10x bar = 100µm. **(B)** Analysis of size of the TrkB positive neurons identified from SC nerve fibers in the dorsal horn. C fibers are on average 0.5-2.0 µm in diameter, while Aδ is on average 1-5µm in diameter. NC SC fibers have a mean diameter of 1.12 ± 0.34 µm, compared to AC12 with a mean diameter of 1.27 ± 0.33 µm. Interestingly the nerve fibers from EAE12 animals have a mean diameter of 2.01 ± 0.58 µm which is highly significantly different from the nerve fiber diameter for that of AC12 (***$P < 0.0001$).

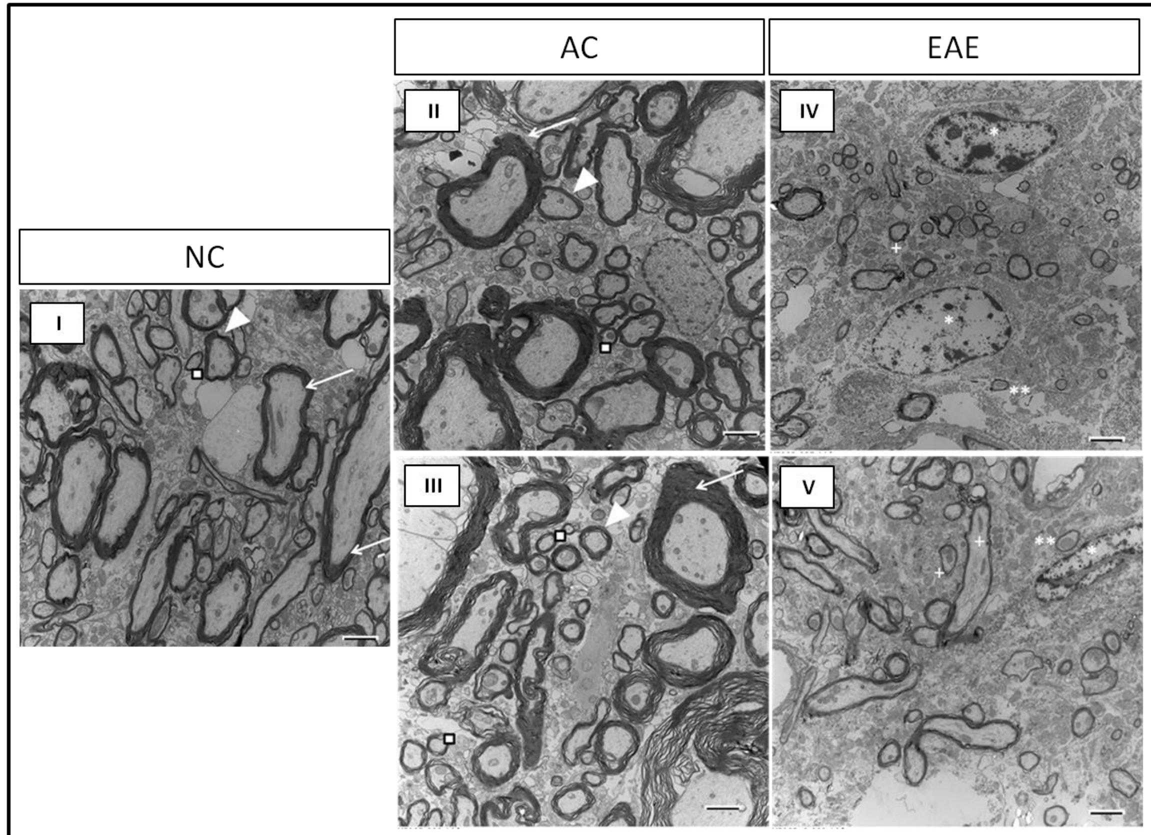

Figure 6: Changes in myelin protein structure and integrity observed by TEM. Direct magnification 4600x using an AMT camera system. Scale bars at 2 µm. The TEM images captured in this figure were obtained from SC tissue subjected to identical experimental conditions and imaging procedures. **I)** SC tissue in NC shows normal myelin ensheathment of various sized axons; myelinated large diameter axons (white arrow), medium diameter (white arrow head), and small diameter axons (white square). **II-III)** There is normal myelin ensheathment in AC12 SC tissue; myelinated large diameter axons (white arrow), medium diameter (white arrow head), and small diameter axons (white square). **IV-V)** TEM images reveal myelin pathology in the SC tissue of EAE12 as demonstrated by the marked reduction of myelin ensheathment in small to

medium diameter axons and an absence of myelin coating in large diameter axons. White** = small diameter non myelinated axons in EAE12 SC tissue. White + = thinly myelinated small to medium diameter axons in EAE12 SC tissue. White * = necrotic cells identified in EAE12 SC.

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PROLOGUE TO CHAPTER 5: RATIONALE FOR MANUSCRIPT

We recently published a conceptual model describing how TNF α, BDNF, and NGF form a critical signaling triad that may ultimately regulate myelin repair [1]. Through our earlier work, we have already demonstrated significant increases in TNF α and BDNF expression during EAE in DRG and SC tissues [2, 3]. However, NGF signaling involving the DRG/SC pathway has yet to be investigated. Substantial evidence indicates NGF has several beneficial actions including immune system regulation [4-9], neuroprotection [10-14], and remyelination [15-17]. Further, several studies in animals with EAE and patients with MS support the beneficial role of NGF [18-22]. Currently, the indirect effects of inflammatory cytokines on DRG and SC derived NGF have not yet been studied as a plausible mechanism of myelin repair. Immune-mediated inflammation is a major contributor to myelin damage [23]. Exploring the changes in NGF expression during the earlier stages of disease may provide crucial information regarding its potential use as a therapeutic agent to ameliorate inflammatory processes that ultimately contribute to myelin destruction.

Based on this rationale, we conceived a study to determine the gene and protein expression changes of NGF during acute, inflammatory, pre-demyelinating EAE. By identifying early changes in NGF signaling future MS therapies may be able to target earlier events to prevent permanent damage. In addition, this study may provide information pertinent to reversing permanent disability.

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CHAPTER 5: NERVE GROWTH FACTOR GENE AND PROTEIN EXPRESSION INCREASES DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: IMPLICATIONS IN MULTIPLE SCLEROSIS AND MYELIN REPAIR

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STATEMENT OF CONTRIBUTION:

For this study, I functioned as the lead investigator. I was responsible for organizing and performing all the animal studies, including EAE induction, animal monitoring, and tissue sample collection and preparation for subsequent analyses. Further, I established the standard operating procedures (SOP) or protocols for the following technical aspects of my research which included: IHC, western blot (WB), ELISA, and qRT-PCR. In addition, I performed all the statistical analysis of the raw data collected. Following the

completion of the data analysis, I was responsible for the inception and design of the manuscript, selection of references, and composing the initial draft. I will further be responsible for copy proofing and revisions of the final manuscript pending submission and acceptance for publication. Overall, I was responsible for establishing all the intellectual decisions in regard to this project following consultation with my primary supervisor, Dr. Namaka.

CHAPTER 5

5.1 ABSTRACT

Multiple sclerosis (MS) is a chronic progressive neurological disease that results in the focal destruction of myelin in the central nervous system (CNS). While the exact mechanism of MS pathology is unknown, it is widely accepted to involve immune-mediated processes, which contribute to inflammation, oligodendrocyte (OG) death, myelin destruction, and neurodegeneration. Recently, studies aimed at identifying the role of NGF in MS offer potential insights into new targeted treatment strategies for this disease because of its ability to modulate immune-mediated processes, promote the survival of OGs and neurons, and support re-myelination. However, the role of NGF is still not completely understood. We used an experimental autoimmune encephalomyelitis (EAE) animal model of MS in adolescent female Lewis rats to study time and disease state dependent changes in NGF expression. Neurological disability scores (NDS) were quantified and correlated to changes in NGF expression in dorsal root ganglia (DRG) and spinal cord (SC) tissues, harvested throughout disease progression. Peak NDS was shown to occur at day 13 post-EAE induction. Overall, we have shown that NGF mRNA and protein expression were significantly increased in DRG and SC tissues in animals induced to a state of EAE compared to healthy and non-disease controls. In the DRG, the increase in NGF mRNA and protein occurred during the time period associated with complete neurological recovery, at days 18 to 24 post-EAE induction. In comparison, NGF protein expression in the SC was increased at day 12 post-EAE induction, which suggests there is an acute local release of NGF from

surrounding supportive cells in the SC which may act to suppress the disability induced by EAE. Interestingly, the increase in SC NGF mRNA expression at day 15 post-EAE induction occurred after the increase in NGF protein expression at day 12 post-EAE induction. We believe this delayed increase in NGF mRNA expression at day 15 post-EAE to be an essential compensatory mechanism required to replenish the immune system–mediated acute release of NGF from the localized supportive cells of the SC which would be essential for the initiation of the remission/recovery phase of EAE. Although, we identified the presence of several high molecular weight isoforms of NGF including the 25 kDa pro-NGF isoform, we could not detect the mature isoform of NGF (13 kDa). In the SC, we observed through transmission electron microscopy (TEM) a thinning and loss of myelin ensheathment in various size axons of animals at day 12 post-EAE induction that correlated with maximal neurological disability.

Our results support the role for NGF in promoting neurological recovery following EAE induction. Our findings suggest that NGF may act as an "off switch" for immune system-induced pathological induction of a cytokine-neurotrophin (NT) signaling triad that governs the degree of myelin damage. Henceforth, our study supports the role of centrally derived NGF in minimizing inflammation and myelin damage associated with EAE induction. The results of our findings are also consistent with our previous publications showing TNF α and BDNF are also up-regulated in the SC during peak neurological disability. In addition, our study also identifies the 25 kDa pro-NGF isoform as being a biologically active form of NGF that becomes activated following EAE

induction. Finally, our research also demonstrates that during the inflammatory pre-demyelinating phase of EAE, the ultrastructural integrity of myelin is already compromised. As such, this supports the concept that early treatment is essential to slow disease progression and minimize the neurological disabilities associated with MS. This study provides novel information relating to the role of NGF during acute phase inflammatory EAE that may be useful in the development of future MS therapies.

Keywords: Multiple sclerosis, MS, EAE, nerve growth factor, NGF, DRG, spinal cord

5.2. INTRODUCTION

Multiple sclerosis (MS) is a chronic, progressive, inflammatory disease of the central nervous system (CNS) that is characterized by immune-mediated myelin damage, axonal demyelination, and neurodegeneration [1-3]. While the exact cause and underlying cellular and molecular mechanisms remain elusive, it is widely accepted to be an autoimmune disease. According to this view, $CD4^+$ Th1 cells are activated upon exposure to an antigen resembling CNS myelin proteins [4], such as myelin basic protein (MBP) [5], proteolipid protein (PLP) [6], and myelin oligodendrocyte glycoprotein (MOG) [7]. These myelin reactive $CD4^+$ Th1 cells cross the blood-brain barrier into the CNS to promote the sustained production and liberation of inflammatory cytokines, such as tumor necrosis factor α (TNF α), interferon-γ (IFNγ), interleukin (IL) -1, -6 and -12 [8-13] that results in the targeted, immune-mediated destruction of myelin.

Demyelination is highly conducive to processes leading to axonal degeneration that cause permanent neurological deficits [14-17] and is unaffected by current MS treatments, which are largely immunomodulatory. Therefore, a dual approach to therapy that includes immunomodulatory treatments, in conjunction with re-myelinating therapeutics, should be employed. One strategy would be to suppress neuroinflammation and promote re-myelination via neurotrophin based treatments. An attractive candidate for novel anti-neuroinflammatory and re-myelinating treatments for MS is nerve growth factor (NGF). While, NGF plays a critical role in the development and maintenance of sympathetic and

sensory neurons and maintenance of cholinergic neurons [18-23], it more recently has gained considerable interest due to its immunosuppressive [24-27] and immunomodulatory functions [25, 28, 29]. NGF can interfere with the immune responsiveness of microglial cells by suppressing IFN-γ-dependent induction of major histocompatibility complex (MHC) II [24]. In addition, the release of NGF from genetically modified T cells reduces T cell-mediated CNS inflammation [27] and infiltration of inflammatory cells [26]. NGF can further induce anti-inflammatory effects by down-regulating the production of T cell derived IFN-γ and up-regulating glial cell production of IL-10 in EAE lesions [25].

NGF is neuroprotective [30-34], enhances axonal regeneration [35-37] and promotes axonal branching and elongation [38], which are integral steps required for re-myelination [39]. Oligodendrocytes (OGs), the myelin producing cells of the CNS, are important for axonal support and viability of myelinated axons [40-42]. NGF can induce myelination in the CNS [43, 44], which may reflect its ability to regulate myelin protein expression in OGs [45, 46], regulate OG process regeneration and proliferation [47], and promote the protection and survival of OGs and oligodendrocyte precursors (OPs) [44, 47, 48]. More specifically, NGF protects OGs from TNF α-mediated apoptosis via the Akt pathway [48] required for OG survival [49]. This demonstrates a critical function of NGF in myelination because TNF α is a major contributor to demyelination, OG damage, and OG apoptosis [50]. TNF α-induced apoptosis and demyelination [50] is mediated through TNF receptor 1 (TNFR1) [51, 52]. However, in the presence of NGF [48], TNF

α can promote OP proliferation and myelination through TNF receptor 2 (TNFR2), though this mechanism is still unknown [50, 53, 54]. Thus, preferential TNF α signaling via TNFR2 results in a protective rather than pathogenic role for TNF α [55, 56]. TNF α can also induce the expression of NGF [57, 58] and another neurotrophin, brain-derived neurotrophin factor (BDNF) [53, 59] **(Fig. 1)**.

NGF may affect re-myelination indirectly through its ability to alter the expression levels BDNF [55, 60, 61]. In mice induced to a state of EAE, BDNF administration resulted in a significant increase in re-myelination [62]. BDNF can promote OP proliferation [63, 64], OG differentiation, and OP development via the TrkB/MAPK pathway [46, 65, 66]. BDNF further plays an integral role in myelin formation [67, 68] and can increase myelin protein expression [65, 69, 70]. NGF has been shown to induce BDNF gene expression in dorsal root ganglia (DRG) neurons [60, 61]. BDNF has also been shown to induce NGF secretion [71]. In addition, BDNF can mediate its biological effects through the NGF receptor, TrkA [61, 72, 73]. Therefore, a potential reciprocal and synergistic relationship between NGF and BDNF may regulate myelin repair. Interestingly, BDNF is transcriptionally repressed by methyl CpG Binding Protein 2 (MeCP2) [74, 75], which is expressed in both neuronal and glial cells [76, 77]. As such, MeCP2 may *indirectly* regulate myelin formation *via* transcriptional suppression of BDNF, thus ultimately influencing NGF and TNF α expression, thereby, disrupting the homeostatic signaling between cytokines and neurotrophins that are known to govern re-myelination and/or myelin repair.

Our recently published model of MS induction describes the role of immune system-mediated DRG activation as essential to promoting a critical signaling triad involving TNF α, BDNF, and NGF that ultimately regulates myelin repair in the spinal cord (SC) [78]. In addition, we have now included MeCP2 as a key upstream regulator of this cytokine-neurotrophin signaling triad. Previously, our research group confirmed the involvement of the dorsal roots in the transport of BDNF from the DRG to the SC [79] that may have implications for re-myelination and/or myelin repair. Furthermore, our previous findings demonstrated that the EAE induced BDNF that is ultimately transported to the SC is produced by small to medium sized sensory neurons housed within the DRG [79]. As such, we have designed the current experiment to further investigate the role of NGF in the amelioration of inflammatory processes contributing to myelin damage. We hypothesized that *NGF gene and protein expression is elevated within the DRG and SC during the inflammatory stage of EAE which facilitates neurological recovery following EAE-induced myelin damage.*

This study is the first, to our knowledge, to demonstrate significant temporal elevations in NGF gene and protein expression in the DRG and SC of rats induced to a state of EAE. Furthermore, this study that identifies (*via* TEM), compromised myelin structural integrity during the early inflammatory phase of the disease prior to demyelination. Our study provides new insights into the role of NGF regulating the repair of immune-mediated myelin damage and potential benefits as a novel therapeutic agent for MS.

5.3. MATERIALS AND METHODS

5.3.1 INDUCTION OF EXXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis (EAE) was induced in adolescent (6 week old) female Lewis rats with MBP (Charles River, Montreal, Quebec, Canada) as previously described [80]. Animals (n = 105) were randomly divided into three experimental groups: *naïve control* (NC), *active control* (AC) and *EAE*. The NC group (n =7) did not receive any treatment. The AC (n =49) and EAE (n = 49) groups were assessed daily until sacrificed for neurological disability. The experimental groups were sacrificed at 3, 6, 9, 12, 15, 18, 21, and 24 days post induction (dpi). All animal experiments in this study were conducted according to protocols approved by the University of Manitoba Animal Protocol Management and Review Committee and in full compliance with the Canadian Council on Animal Care.

5.3.2. COLLECTION AND PREPARATION OF DRG AND SC TISSUES

DRG and SC tissues for immunohistochemical (IHC) analysis of protein expression were collected from 48 animals (AC: n = 24, EAE: n =24) that were perfusion fixed with 4% paraformaldehyde, according to our previous publications [80], at the described pre-determined days with 3 additional animals from the NC group being perfusion fixed at 12

dpi. Whole spinal columns were subsequently decalcified with 6% trichloroacetic acid according to previously described protocols [81]. Cryoprotected lumbar DRG and spinal cord tissues were sectioned at a 10 µm thickness and mounted on charged slides (Fisher Scientific, CN: 12-550-15) in groups of 10 with 3 sections per slide. In addition, spinal cord tissues from each experimental group (n =1) were perfusion fixed at 12 dpi with 1.5% glutaraldehyde (E.M.S, CN: 16210) in 0.1 M Sorensen's phosphate buffer, pH 4. These tissues were cut into 1 mm by 1 mm sections and drop fixed in the same fixative solution for 3 hours, after which the fixative was removed and replaced with 5% sucrose in 0.1 M Sorensen's buffer for 1 hour. Post-fixation was performed using 1% osmium tetroxide before embedding in Epon (E.M.S., Embed-812 embedding kit, CN: 14120). For quantitative gene and protein analysis, the DRG and spinal cord were harvested from 51 animals (NC: n = 3, AC: n = 24, EAE: n =24) within 30-45 minutes from the time of death at the described pre-determined days with NC animals being sacrificed at 12 dpi. Tissues were stabilized in RNAlater (Qiagen, CN: 76106) until processed for extraction. Total RNA, DNA, and protein were purified simultaneously as described previously [82].

5.3.3. QUALITATIVE IMMUNOHISTOCHEMICAL (IHC) PROTEIN ANALYSIS

Qualitative IHC analysis of cryostat sections was performed to detect NGF protein expression in the lumbar DRG and spinal cord. Immunofluoresence was achieved using a rabbit polyclonal IgG against NGF (Santa Cruz, CN: SC-549). The anti-NGF antibody

detects the 13 and 25 kDa isoforms of NGF. The secondary antibody used against the anti-NGF antibody was TRITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, CN: 711-025-152). The slides were washed with PBS three times at 5 minute intervals, blocked with 15% normal donkey serum (Sigma, CN: D9663-10ML) in 0.3% PBS-T for 1 hour, washed three times in PBS, and then incubated with the primary antibody against NGF (1:500) in 5% normal donkey serum in 0.15% PBS-T overnight at 4°C. Following incubation with the primary antibody, the slides were washed tree times in PBS and incubated with the secondary antibody (1:100) in 5% normal donkey serum in 0.15% PBS-T for 1 hour. The slides were washed three times in PBS and one drop of mounting medium (EMS, CN: 17985-01) was added to tissues and subsequently protected with a cover slip. Imaging was performed using the Nikon Eclipse Ti-S fluorescent microscope equipped with an epi-fl illuminator. Image sizing, black background balancing and final collation for publication were performed using Adobe PhotoShop CS2 (Adobe Systems Inc.). No image manipulations were performed other than those described.

5.3.4. SEMI-QUANTITATIVE WESTERN BLOT (WB) PROTEIN ANALYSIS

Lumbar DRG and caudal spinal cord tissues were homogenized and total supernatant protein concentration was determined using a BCA protein assay kit (Novagen, CN: 71285-3). For each sample, 20 μg total protein was separated by 4-20% Tris-Glycine gradient SDS-PAGE (Thermoscientific, CN: 0025269) at 120 V for 1 hour and

electrophoretically blotted onto a PVDF membrane (Immobilon, CN: IPFL00010) for 1 hour at 0.35A. An internal standard was similarly loaded in order to normalize between membranes. The membranes were blocked in 5% skim milk in TBS-T for 1.5 hours and incubated with primary rat monoclonal IgG (1:500, Promega, CN: G1132) against NGF or rabbit polyclonal IgG against GAPDH (1:1000, Santa Cruz, CN: sc-25778) overnight at 4ºC. GAPDH was utilized as a loading control to ensure equivalent amounts of protein were loaded and normalize NGF protein densities. The primary anti-NGF antibodies recognize the 25 kDa NGF isoform and anti-GAPDH antibodies detect 37 kDa GAPDH. After incubation with primary antibodies the membrane was washed with TBS-T and incubated with secondary antibodies. The secondary antibodies used were peroxidase-conjugated goat anti-rat IgG (1:1000, Jackson Immunoresearch, CN: 112-035-175) against the anti-NGF antibody and peroxidase-conjugated mouse anti-rabbit IgG (1:10,000, Jackson Immunoresearch, CN: 211-032-171). The antigen-antibody complexes were detected using ECL detection reagent (Pierce, CN: PI32209) Membranes were exposed to chemiluminescence for 30 minutes and densitometry was performed using a FluorChem 8900 scanner (Alpha Innotech) with Alpha Ease FC software. Densitometry analysis was conducted with ImageJ. The individual NGF and GAPDH band densities for each sample were normalized to the internal standard. The sample and loading control density ratios obtained relative to the internal standard were subsequently used to calculate the relative density ratio of NGF relative to GAPDH.

5.3.5. QUANTITATIVE ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

Total protein was extracted and protein concentration was determined as described above. Protein concentration was adjusted to 10 µg in 100 µl total volume. Sandwich format enzyme-linked immunosorbant assay (ELISA) was performed using an NGF Emax ImmunoAssay System (Promega, CN: G7631) according to manufacturer's instructions. NGF protein concentration was interpolated from a standard curve with a range of 3.9-250 pg/ml.

5.3.6. QUANTITATIVE REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

Lumbar DRG and caudal spinal cord tissues were homogenized and total supernatant RNA concentration was calculated from its absorbance. Reverse transcription was performed with varying amounts of total RNA and qPCR cDNA synthesis kit (Agilent, AffinityScript qPCR cDNA synthesis kit, CN: 600559) in a total volume of 20 ul and according to the manufacturer's instructions. Real-time PCR was performed with the CFX96 real-time PCR detection system (Biorad) using SYBRGreen (Toyobo, Thunderbird SYBR qPCR Mix, CN: QPS-201) following the manufacturer's protocol with designed gene-specific primers and 5 ul of 25 ng RNA equivalent cDNA in a total volume of 20 ul. For NGF the primers were forward, 5'-

CTGTGGACCCCAGACTGTTT-3'; reverse, 5'-TGCTCCTGTGAGTCCTGTTG-3'. For glyceraldehydes-3-phosphate-dehydrogenase (GAPDH), the primers were forward, 5'-AAGAAGGTGGTGAAGCAGGCG-3'; reverse, 5'-AGACAACCTGGTCCTCAGTGTAGC-3'. For quantification, NGF gene expression was normalized to the housekeeping gene, GAPDH, which is known to be found in all tissue at relatively constant levels. Relative quantification of NGF transcripts was determined using the ΔΔCt method.

5.3.7. TRANSMISSION ELECTRON MICROSCOPY (TEM) ASSESSMENT OF MYELIN STRUCTURE

SC ultrastructure was observed with TEM for changes in myelin protein structure and integrity. SC tissues from each experimental group (n =1) were perfusion fixed 12 dpi with 4% paraformaldehyde. These tissues were cut into 1 mm by 1 mm sections and drop fixed in the same fixative solution for 3 hours, after which the fixative was removed and replaced with 5% sucrose in 0.1 M Sorensen's buffer for 1 hour. Post-fixation was performed using 1% osmium tetroxide before embedding in Epon (E.M.S., Embed-812 embedding kit, 14120). TEM was performed with a Philips CM10, at 60 kV, on ultra-thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate.

5.3.8. STATISTICAL ANALYSIS

Data from all samples analyzed from each animal group are reported as mean ± standard error of the mean (SEM). Data was compared using 2-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Statistical significance was considered to be $P < 0.05$.

5.4. RESULTS

5.4.1. NEUROLOGICAL DISABILITY SCORES (NDS)

Animals induced to a state of EAE were assessed daily for neurological deficits and disability was quantified using a previously described neurological disability assessment scale [80]. None of the animals with EAE exhibited neurological disability prior to 6 dpi (score = 0). The earliest onset of neurological disability was observed at 7 dpi (mean 0.011 ± 0.011), as animals entered the induction phase of EAE. Neurological disability became progressively worse before peaking at 13 dpi (3.035 ±0.521). These animals entered the remitting phase of EAE at 14 dpi (2.699 ± 0.463) and exhibited complete neurological recovery by 19 dpi (score = 0) **(Fig.2)**. None of the animals in the NC and AC groups displayed neurological symptoms.

5.4.2. IHC ANALYSIS OF NGF PROTEIN EXPRESSION IN DRG TISSUE

To view the spatial and temporal distribution of NGF protein we used IHC to qualitatively analyze NGF protein expression. In the DRG, comparative IHC analysis of NC, AC, and EAE animals, revealed a marked increase in NGF immunoreactivity in DRG neurons in the EAE group compared to NC and AC at 21 and 24 dpi (**Fig. 3**). This increase in NGF expression is observed occurred during the remission/recovery stage of EAE. Our results provide evidence to support the concept that NGF protein expression peaks in the DRG during complete neurological recovery following EAE induction.

5.4.3. WB ANALYSIS OF NGF PROTEIN EXPRSSION IN THE DRG

Additional protein detection assays were employed to support the IHC data and to determine time and disease state dependent changes in NGF protein expression. Using two different polyclonal anti-NGF antibodies (Santa Cruz, sc-549 and sc-548), each specific for pro-NGF and mature NGF, we attempted to detect specific immunoprecipitant bands corresponding to 25 kDa pro-NGF and 13 kDa mature NGF. Interestingly, in the DRG both antibodies utilized detected larger molecular weight isoforms between 25 and 100 kDa *(data not shown).* There was sporadic detection of the 25 kDa isoform using one antibody (Santa Cruz, sc-549) and no detection of the 25 kDa isoform with the other (Santa Cruz, sc-548) *(data not shown).* The 13 kDa isoform was undetectable in all samples using both antibodies *(data not shown).* Subsequently, a

monoclonal anti-NGF antibody capable of detecting only pro-NGF (25 kDa - Promega, G1132) was employed. A specific immunoprecipitant band was revealed at approximately 25 kDa, corresponding to pro-NGF **(Fig. 4)** in all animals in each group thereby confirming the specificity of the antibodies used for ELISA. Our WB results also confirmed a notable increase in NGF protein in the DRG at 21 and 24 dpi in the EAE group that was identified in our IHC analysis (**Fig. 3**).

5.4.4. NGF PROTEIN EXPRESSION IN THE DRG USING ELISA

To further pursue the NGF protein analysis beyond that depicted in our IHC (**Fig. 3**) and WB (**Fig. 4**) data, quantitative ELISA was employed to conclusively determine the differential protein expression for NGF amongst the experiemental animal groups at the outlined experimental time points. For each sample results are given as pg NGF per 10 μg total protein. In the DRG, ELISA revealed a significant increase in the expression of the 25 kDa pro-NGF in animals with EAE at 21 and 24 dpi, which correlated with complete neurological recovery **(Fig. 2).** NC animals (white bar) (**Fig. 5**) show NGF protein expression as 5.180 pg ± 0.672/10 μg of total protein. In AC animals at 21 and 24 dpi (grey bars) the mean NGF protein expression was 3.198 ± 0.156 and 3.275 ± 0.910 pg/10 μg of total protein, respectively. In animals induced to state of EAE, NGF protein expression reaches maximal levels during the time of neurological recovery at days 21 and 24 dpi. These changes in NGF expression are significantly increased in animals with EAE at 21 dpi [mean 14.166 ± 2.796 pg/10 μg of total protein; $P < 0.0001$

(NC) and $P < 0.0001$ (AC 21 dpi)] and 24 dpi [mean 12.287 ± 2.705 pg/10 μg of total protein; $P < 0.005$ (NC) and $P < 0.0001$ (AC at 24 dpi)] animals (black bars) compared to NC, AC animals at 21 and 24 dpi **(Fig. 5)**. There were no significant differences between NC and AC at all time points.

5.4.5. NGF GENE EXPRESSION IN THE DRG

Comparative quantitative real-time PCR was conducted to support the confirmed results obtained from our protein detection assays (IHC, WB and ELISA). Specifically, we wanted to determine if the changes in protein expression are also associated with a corresponding translational increase in mRNA expression. Results are shown as NGF mRNA expression normalized to GAPDH in AC and EAE animals relative to NC. The mRNA expression of GAPDH was consistent across all samples in DRG throughout all experimental time points. Quantification of NGF mRNA expression within the DRG identified significant increases in EAE animals at 18 and 21 dpi, which correlated with complete neurological recovery **(Fig. 2)**. In AC animals (grey bars) mRNA expression remains relatively unchanged throughout time post-induction. The mean fold increase in NGF mRNA expression in the AC animals at 18 and 21 dpi were 0.517 ± 0.093 and 0.380 ± 0.059, respectively. The NGF mRNA expression is significantly increased in EAE animals at 18 dpi (mean 1.610 ± 0.095; $P < 0.05$) and 21 dpi (mean 1.567 ± 0.311; $P < 0.05$) animals (black bars) compared to AC animals at 18 and 24 dpi **(Fig. 6)**. There

are no significant differences between NC and AC in all predetermined experimental time points except 24 dpi ($P < 0.05$) *(data not shown).*

5.4.6 IHC ANALYSIS OF NGF PROTEIN EXPRESSION IN SC TISSUE

Comparative IHC analysis of NC, AC, and animals with EAE, revealed a marked increase in NGF immunoreactivity in EAE animals at 12 dpi throughout the white and grey matter of the SC compared to NC and AC at 12 dpi (**Fig. 7**). More intense NGF immunoreactivity can be observed in the grey matter of the SC. This increase in NGF expression produced by local SC supportive cells seen during peak neurological disability occurs in an attempt to counteract the EAE induced neurological deficits. As such, the transient increase in NGF protein peaks in the SC at 12 dpi is represents the "off switch" to counter the negative effects that arise from the EAE induced immune system insult.

5.4.7 WB ANALYSIS OF NGF PROTEIN EXPRESSION IN THE SC

In the SC, larger molecular weight isoforms between 25 and 100 kDa *(data not shown)* were detected using both polyclonal anti-NGF antibodies (Santa Cruz, sc-549 and sc-548) specific for both the 25 kDa pro-NGF and 13 kDa mature NGF. Similar to the DRG tissue, the 25 kDa isoform was sporadically detected using one antibody (Santa Cruz, sc-549) and scarcely detectable using the other (Santa Cruz, sc-548) *(data not shown).* The 13 kDa isoform was undetectable in all samples using both antibodies *(data not shown).*

When the monoclonal anti-NGF antibody specific for pro-NGF (Promega, G7631) was utilized, a specific immunoprecipitant band was revealed at approximately 25 kDa, corresponding to pro-NGF in all animals in each group **(Fig.8).** Quantification of NGF protein by WB densitometry is reported as NGF/GAPDH ratio. Densitometry analysis showed the mean NGF protein expression in the NC to be 0.533 ± 0.141, 0.450 ± 0.023 in AC animals at 12 dpi, and 1.523 ± 0.246 in EAE animals at 12 dpi. The EAE 12 dpi animals had a significant increase in NGF protein expression ($P < 0.01$; $P < 0.001$, respectively) compared to NC and AC at 12 dpi **(Fig. 9).** There are no significant differences between the NC and AC groups at all experimental time points.

5.4.8. NGF PROTEIN EXPRESSION IN THE SC USING ELISA

NGF protein expression in the SC was increased at 12 dpi which indicates a plausible acute local release of NGF from surrounding supportive cells in the SC in attempt to suppress the disability induced by the EAE insult. Specifically, our ELISA results demonstrated a significant increase in 25 kDa pro-NGF protein expression in animals with EAE at 12 dpi (**Fig. 10**). Results are given as pg NGF per 10 μg total protein. NGF protein expression was 1.431 ± 0.113 pg/10 μg of total protein in NC animals (white bar). In the AC group (grey bars) the mean NGF protein expression remains relatively unchanged post-induction. The mean NGF protein expression was 0.804 ± 0.432 pg/10 μg of total protein in AC animals at 12 dpi. During the disease course, there is a gradual increase in SC NGF protein expression that reaches maximum expression at 12 dpi. This

peak increase in NGF is significant in animals with EAE at 12 dpi [mean 3.052 ± 0.607 pg/10 μg of total protein; $P < 0.05$ and $P < 0.001$, respectively) animals (black bars) compared to NC and AC animals at 12 dpi **(Fig. 10)**. There are no significant differences between NC and AC at all time points.

5.4.9. NGF GENE EXPRESSION IN THE SC

Interestingly the increase in SC NGF mRNA expression at 15 dpi (**Fig. 11**) occurred after the increase in NGF protein expression (12 dpi) (**Fig. 10**). However, we suggest this delayed increase in NGF mRNA expression at 15 dpi to be an essential compensatory mechanism required to replenish the immune system-mediated acute release of NGF protein at 12 dpi from the supportive cells of the SC (**Fig. 10**). As such, this delayed increase in NGF mRNA in the SC at 15 dpi appears to be essential for the initiation of the remission/recovery phase of EAE, which may serve as an “off switch” for the immune system mediated insult on SC myelin.. Results are shown as NGF mRNA expression normalized to GAPDH in AC and EAE animals relative to NC. The mRNA expression of GAPDH was consistent across all samples in SC at all experimental time points. The mRNA expression in AC animals (grey bars) remains relatively stable throughout time post-induction. The mean fold increase in NGF mRNA expression in the AC animals at 15 dpi was 0.899 ± 0.147. In comparison, in the EAE group NGF mRNA expression gradually increased in parallel with neurological disability and reached maximum levels after peak neurological disability (13 dpi), before returning to normal levels during

remission **(Fig.2).** The peak levels in NGF mRNA expression identified in animals with EAE at 15 dpi (mean 3.675 ± 1.999; $P < 0.05$) is significantly increased compared to AC animals at 15 dpi **(Fig. 11)**. There were no significant differences between NC and AC at all other time points *(data not shown).*

5.4.10. SC MYELIN PATHOLOGY IN THE SC OBSERVED IN TEM

Lumbar SC ultrastructure was observed using TEM to assess the effect of EAE disease on the structural integrity of SC myelin. Comparative assessment of SC TEM images for NC, AC animals at 12 dpi, and EAE animals at 12 dpi revealed significant myelin pathology in animals with EAE **(Fig.12 I-V)**. TEM images of the SCs of NC and AC animals at 12 dpi reveal normal myelin ensheathment of various sized axons with no differences between the two groups **(Fig. 12 I, II, III).** However, myelin is notably thinner in animals with EAE at 12 dpi **(Fig. 12 IV, V)** than in both controls **(Fig. 12 I, II, III).** The myelin sheath surrounding the axons of small to medium diameter neurons at 12 dpi in animals with EAE are markedly thinner. In addition, the EAE animal at 12 dpi showed a pronounced absence of myelin ensheathment in large diameter axons, as well as, the existence of necrotic cells. These changes in myelin integrity correlated with peak neurological disability at 13 dpi **(Fig. 2)**.

5.5. DISCUSSION

While there are several disease-modifying treatment options available for MS, no real significant advances have been made in developing re-myelinating and/or myelin repair therapies. This is largely due to the fact that the conditions required to promote myelin re-myelination and/or repair are not fully understood. NGF has garnered a great deal of attention for its ability to repair myelin damage and reverse neurological disability [57, 83, 84]. Although, several studies support the beneficial role of NGF in myelin repair, the exact role of NGF in MS is not fully understood and requires further investigation. MS research has confirmed that immune system-mediated inflammation is a major contributing factor to myelin damage and demyelination [85]. Given the supportive evidence in regard to the immunomodulatory role for NGF, it is imperative to investigate its role during the early acute inflammatory phase of the disease prior to demyelination. Optimizing the beneficial effects of NGF at the early onset of disease may be an integral step in preventing myelin damage even prior to its occurrence.

We previously published TNF α and BDNF are both significantly increased during EAE at 12 dpi in the DRG [79, 80]. Our current findings are consistent with our previously published research in this area and the current literature that supports the cytokine-neurotrophin signaling system in regard to the DRG-SC connective pathway [39, 53, 55, 56, 61, 86-90]. In the presence of NGF, TNF α preferentially signals through TNFR2 leading to protective rather than pathogenic effects on myelin [48]. However, in the

absence of sufficient levels of NGF, TNF α preferentially signals through the TNFR1 pathway to drive inflammation and worsen the immune system-mediated attack on myelin [91, 92]. Based on our previous publication, DRG-derived TNF α may be subsequently transported from the DRG into the SC following an EAE insult. [80]. As such, TNF α can mediate myelin damage in the SC. However, what we do know, is that TNF α also induces the expression of BDNF [87, 93] and that BDNF is a critical factor involved in re-myelination and/or myelin repair [64, 94-99]. Specifically we have published that following EAE induction, TNF α induces sensory neurons of the DRG to produce BDNF. BDNF is subsequently anterogradely transported via the dorsal roots into the SC [79]. We have also shown that this increase in DRG-derived BDNF within the SC is directly correlated to neurological recovery/remission of the disease [79]. Interestingly, the literature also indicates that BDNF induces NGF secretion [71]. As such, we designed a study to determine the temporal changes in NGF expression during the disease course in an MBP-induced EAE model of MS. Therefore, our current study tested our hypothesis that elevated levels of NGF in the DRG and SC during the early inflammatory stage of EAE contributes to neurological recovery and ultimately, myelin repair.

5.5.1. NGF GENE AND PROTEIN INDUCTION CHANGES IN THE DRG DEMONSTRATE TISSUE AND TIME DEPENDENT RESPONSES DURING DISEASE AND RECOVERY PHASES OF EAE

Our findings demonstrated NGF gene and protein expression is up-regulated in the DRG of animals induced to a state of EAE during the complete neurological recovery/remission phase of the disease. Comparative assessment of the spatial and temporal distribution of NGF identified the source of NGF to be the sensory neurons housed within the DRG. Additional, protein quantification methods corroborate these significant increases in NGF protein expression in the EAE group during complete neurological recovery in the DRG. As expected, the increases in protein expression (in animals with EAE at 21 and 24 dpi) are preceded by the translational increase in NGF mRNA (at 18 and 21 dpi). EAE animals at 21 dpi demonstrated an increase in NGF mRNA which corresponded with an increase in NGF protein. Studies suggest neurotrophins, such as BDNF [79], signal anterogradely [100]. However, the prevailing concept for NGF is that it is produced in peripheral and central target tissues, is internalized in a receptor-dependent manner through TrkA, and transported *retrogradely* from distal axons to the neuronal cell body to control survival, growth, and differentiation [101, 102]. Despite this, recent, but limited, evidence suggests NGF may also be anterogradely transported [103]. Therefore, we believe this sequential up-regulation of NGF mRNA and protein suggest that the DRG may serve as a secondary compensatory source of NGF for subsequent delivery to the SC where it can assist DRG and locally

derived BDNF in promoting neurological recovery [79]. As such, DRG-derived NGF may act in the SC as a regulator of immune system-mediated insult by also serving as an "off switch" for the initial inflammatory response initiated by inflammatory mediators, such as TNF α [80, 82, 104]. In addition, NGF may serve locally in the DRG as an "off switch" for cytokine (TNF α) and neurotrophin (BDNF) signaling to the SC. We have also shown that this increase in SC BDNF via the DRG is directly correlated to neurological recovery/remission of the disease [79]. Therefore, we suggest a significant increase in NGF expression in the DRG acts as an "off switch" for pathogenic TNF α (promoting protective signaling via TNFR2 in the presence of NGF [48]), thereby sustaining the remitting phase of EAE. Likewise, BDNF signaling would also be turned down, effectively decreasing its unnecessary supply to the SC once remission has taken place. Thus, DRG-derived NGF contributes to neurological recovery/remission following an EAE-mediated insult. This further suggests NGF may play a role in *maintaining* the remitting phase. As such, earlier intervention aimed at the interconnected cytokine-neurotrophin signaling triad [39, 53, 55, 56, 61, 78, 86-90] **(Fig. 1)** in the DRG represents a plausible approach to minimize or prevent myelin damage and/or promote myelin repair.

5.5.2. NGF GENE AND PROTEIN INDUCTION CHANGES IN THE SC DEMONSTRATE TISSUE SPECIFIC RESPONSES DURING THE DISEASE PHASE AND THE ONSET OF NEUROLOGICAL RECOVERY IN EAE

Elucidating the expression changes of NGF in the SC is critical in investigating the functional role of NGF in promoting neurological recovery. Comparative assessment of the spatial and temporal distribution of NGF identified marked increases in NGF expression throughout the SC, particularly in the grey matter during peak neurological disability. Our results, are consistent with that of other researchers that have shown that NGF mRNA, protein, , as well as $p75^{NTR}$ mRNA, are up-regulated during the acute phase of EAE [105]. Our results are also consistent with the human studies that have shown increased levels of NGF in the cerebrospinal fluid (CSF) of MS patients during acute attacks that significantly decrease during remission [106]. Collectively, these findings that depict a transient increase in NGF during an acute attack provide support that NGF is involved in neurological recovery/remission following an immune system-mediated attack on CNS myelin. Our IHC analysis identified DRG sensory neurons as an integral cellular source for NGF that may be utilized to facilitate re-myelination and/or myelin repair. Although we did not conduct co-localization studies to identify specific cells exhibiting NGF immunoreactivity, glial cells, mast cells, T and B cells have been reported to express NGF in the CNS during EAE [107-109]. In addition to synthesizing NGF, mast cells can further store, and secrete NGF [110, 111]. As such, the acute release of NGF protein that we have shown in the SC at 12 dpi is the result of the NGF liberated

by these surrounding cell types. We previously showed BDNF from the DRG is transported to the SC [79]. This supply of DRG-derived BDNF to the SC may act to dampen TNF α activity by facilitating the localized release of NGF from surrounding supportive cells in the SC, which subsequently preferentially promotes TNF α signaling via the TNFR2 pathway, making it protective rather than pathogenic. This would effectively shut down the immune system-mediated pathogenic activity of TNF α. Thus, the localized increase of NGF in the SC represents a critical step in serving as the "off switch" to reduce the TNF α.-induced inflammatory attack on myelin initiated by mediators. Therefore, NGF is a key factor involved in neurological recovery/remission following an immune system mediated insult. Furthermore, it has been shown that there is an up-regulation of TrkA and $p75^{NTR}$ in spinal cord astrocytes during EAE [112, 113]. In the injured rat SC, NGF is expressed in ramified microglia and a subset of astrocytes [114]. Neurons, several subsets of T cells, and B cells can also express NGF, TrkA, and $p75^{NTR}$ [113, 115, 116]. Mast cells [117], monocytes [118] and macrophages [119] also express $p75^{NTR}$. As a result, the NGF liberated by these surrounding cell types following an immune system-mediated insult may bind to its TrkA receptor located on these various cell types where it can subsequently induce the production of NGF from these cell types thereby creating a positive feedback loop to suppress the inflammatory response. Our gene analysis results in the SC indicated a peak in NGF mRNA in animals with EAE at 15 dpi which occurred after the peak in NGF protein expression at 12 dpi. The delay in the peak gene expression that occurs after the peak protein expression may be explained by a compensatory response by surrounding cells in the SC to restore NGF

protein levels that were acutely liberated during the initiation of the immune system-mediated insult on SC myelin.

This non-sequential induction of NGF mRNA and protein represent what may be disease state and tissue specific differential transcriptional, post-transcriptional, translational, and post-translational regulations of the NGF gene and protein synthesis. Generally speaking, mRNAs are produced at a lower rate than proteins in mammalian cells [120]. For instance, more copies of corresponding protein are produced per mRNA than copies of mRNA per hour [120]. Therefore, it is possible the accumulation of NGF protein is a result of increased translational activity and not transcription. This may represent a plausible novel mechanism by which TNF α, DRG-derived BDNF or other neuroinflammatory mediators regulate NGF protein synthesis. Further studies are required to explore this concept. Despite the transient increase in NGF mRNA expression at 15 dpi, NGF protein does not appear to be further translated to significant levels. This is likely due to a decrease in NGF protein requirement in the SC during the neurological recovery/remission phase of the disease. Alternatively, it may also represent a reservoir of available NGF transcripts for protein translation that can be mobilized in the event of serial antigenic attacks on the CNS. The latter would prove to be a substantial finding, if true, since it would provide insights into this specific EAE model which is a monophasic acute disease course that is self-limiting in Lewis rats [121].

5.5.3. 25 kDa Pro-NGF PROTEIN IS A BIOLOGICALLY ACTIVE ISOFORM DURING EAE

The mature isoform of NGF (13 kDa) was originally thought to be the only biologically active isoform [122]. However, it has been suggested that the pro-NGF (various large molecular weight isoforms including 22 to 53 kDa) may have activity similar to, as well as, distinct from the mature isoform [122-124]. It has also been suggested that the biological outcome may depend on the relative ratios of pro and mature isoforms of NGF [122]. In order to determine which NGF isoforms are biologically active during EAE, WB techniques were employed. Mature NGF could not be detected in all samples in DRG and SC tissue. The limited detection of mature NGF (13 kDa) has also been reported previously [125, 126] and is consistent with a study reporting the pro-NGF as the predominant isoform in the brain and Alzheimer's [125]. This suggests the 13 kDa isoform i) may not be a biologically active isoform specific to the disease (EAE-induced MS); ii) has a shorter half-life than pro-NGF due to either rapid degradation or uptake upon cleavage; or iii) the disease process causes abnormalities in pro-NGF processing possibly. In this study, various NGF isoforms were detected between 25 and 100 kDa in DRG and SC tissue. The glycosylated pro-NGF has been identified as a predominant isoform in DRG neurons [127]. Other studies have also identified high molecular weight glycosylated NGF isoforms in various tissues [127-134]. We used an antibody specific for the 25 kDa pro-NGF isoform to determine expression changes of this form. In the DRG and SC, 25 kDa pro-NGF was detected in all samples confirming the specificity of

the antibody selected for ELISA. In contrast, a significant increase in 25 kDa pro-NGF was observed in the SC consistent with our other protein detection assays (IHC and ELISA). While, the role of the pro-NGF is not well understood, these findings recognize the 25 kda pro-NGF as being a biologically active form of NGF during EAE in the SC.

5.5.4. EAE INDUCES THINNING OF MYELIN ENSHEATHMENT CONSISTENT WITH PEAK NEUROLOGICAL RECOVERY

EAE is the most widely employed animal model of MS [135-137]. The Lewis rat MBP EAE model is the most commonly used model for an acute inflammatory attack [121] but is less useful for studying the extensive demyelination observed in MS since it exhibits little to no demyelination [121, 138-141]. However, it is a very useful animal model to study the early inflammatory changes following antigenic insult on the CNS prior to demyelination. Advanced understanding of the inflammatory processes that leads to myelin damage may provide insight into new treatment strategies aimed at myelin damage prevention. Based on our TEM findings, we demonstrate significant structural abnormalities in EAE animals that are not present in controls. As a result, these abnormalities in the ultrastructure of myelin are responsible for the peak neurological disabilities that we have recorded at the same time (12 dpi). Specifically, our research confirms that SC myelin in EAE at 12 dpi was notably thinner than in healthy and non-disease controls. This novel observation demonstrates that the structural integrity of myelin is already compromised during the early inflammatory stage of EAE. Time

dependant comparative analysis at 12 dpi revealed that the myelin coating surrounding the axons of EAE animals exhibit a marked reduction or even absence of myelin ensheathment in various sized axons. We previously reported that medium Aδ fibers are responsible for BDNF transport into the SC [79]. While a marked thinning of myelin in medium diameter neurons may compromise the structural integrity of Aδ fibers, BDNF transport mechanisms may still be functional during EAE as we have previously published. As such, the elevated production and delivery of BDNF protein to the SC may be integral for the liberation of NGF from surrounding cells in the SC that assists in neurological recovery following immune-mediated inflammation and consequently, myelin damage leading to neurological disability. We can, further, correlate these changes in myelin integrity to the peak neurological disability we observed in our animals. Therefore, the up-regulation of NGF expression at 12 dpi in the SC in animals with EAE may be a critical step in halting the inflammatory immune system-induced myelin damage that is responsible for the neurological disabilities identified in our EAE animals.

5.6. CONCLUSION

Our study provides new insights into the role of NGF in regulating inflammation mediated neurological disability and associated myelin damage during early acute neuroinflammatory EAE. The role of NGF in the DRG during EAE is not yet well understood. However, the role of NGF as an anti-inflammatory mediator in the CNS [83]

may extend to the DRG. We demonstrate significant increases in NGF mRNA and protein levels in the DRG that correlate with neurological recovery, which may suggest an essential role of DRG-derived NGF in the down-regulation of cytokine signaling to prevent sustained TNF α-mediated myelin damage via TNFR1 signaling. This research supports the importance of the anatomical connection between the DRG and SC as a critical pathway for the molecular signaling between neurotrophins and cytokines in regard to CNS myelin repair. Further, as NGF can ameliorate clinical symptoms of EAE [57, 83] and induce myelination in the CNS [43, 142], SC-derived NGF may contribute to the recovery from neuroinflammation induced myelin damage that causes neurological disability. We showed a significant increase NGF protein levels in the SC, which we believe to be released from surrounding supportive cells in the SC to promote recovery from neurological deficits imposed by EAE. Although, more studies are required to determine which supportive cells supply NGF, microglial cells which have been implicated in re-myelination and/or myelin repair [143, 144] have been shown to express NGF [145-147]. Thus, microglial cells may serve as a potential source of NGF protein during an immune system mediated inflammatory insult to the CNS. Our findings provide support for a critical role of DRG and centrally derived NGF in promoting and maintaining neurological recovery. Furthermore, our research supports our previous finding which suggests that DRG-derived BDNF transport to the SC is critical in neurological recovery, possibly through its ability to promote the the secretion NGF protein from surrounding cells in the SC. In addition, our study further recognizes the 25 kDa isoform of NGF as being a biologically active isoform of NGF which renders its

importance in re-myelination and/or myelin repair mechanisms. Further this research confirms by TEM that the structural integrity of myelin is compromised during the neuroinflammatory stage of EAE.

5.7. FIGURES

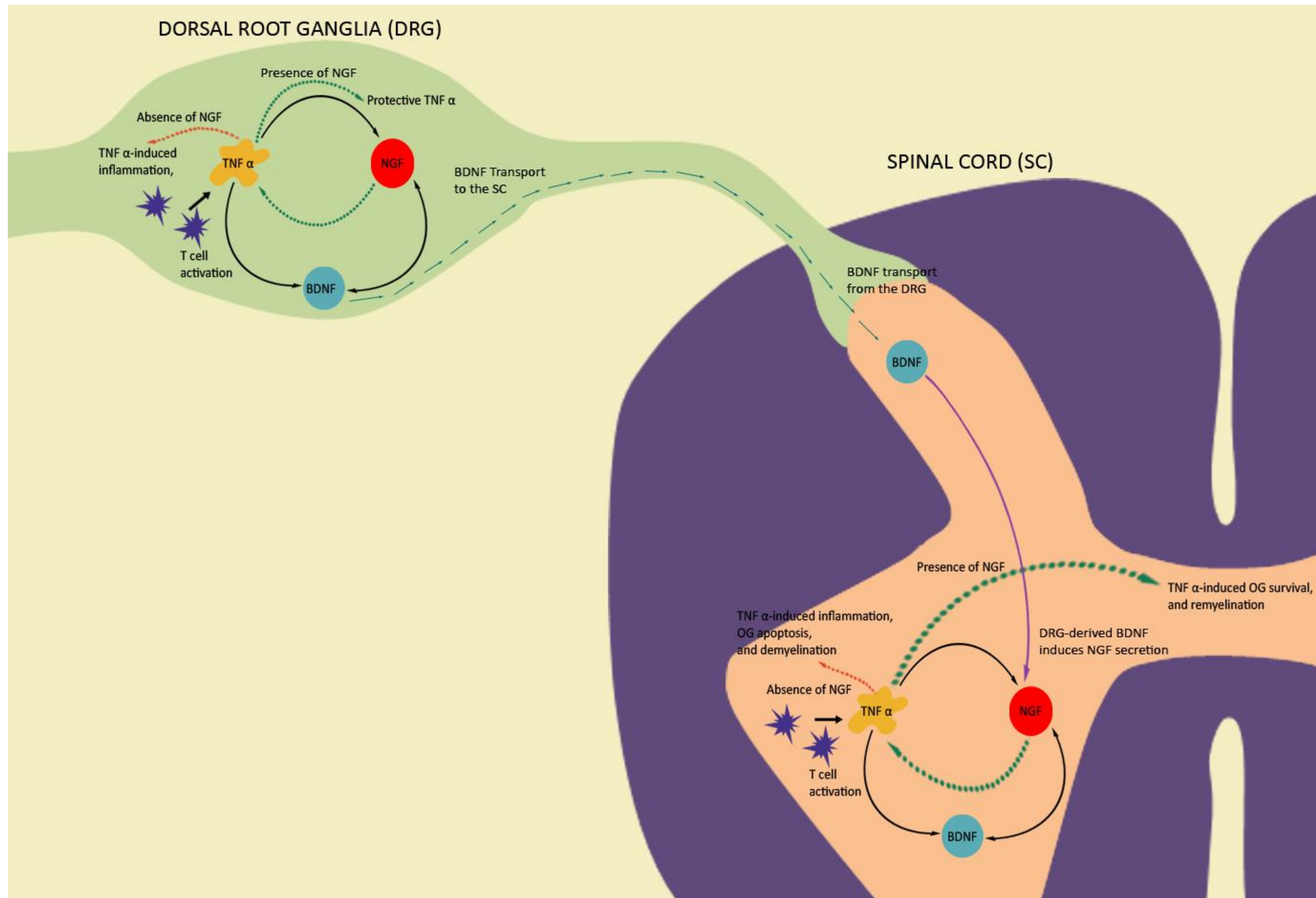

Figure 1. A schematic representation of a proposed cytokine – neurotrophin signaling triad.

T cell activation induces TNF α expression resulting in the induction of BDNF and NGF expression. BDNF can promote NGF secretion and NGF reciprocally induces BDNF expression. In the absence of NGF, TNF α signals through TNFR1 to promote inflammation, OG apoptosis, and demyelination. However, in the presence of NGF, TNF α preferentially signals through a second receptor, TNFR2, to promote re-myelination and the survival of OGs.

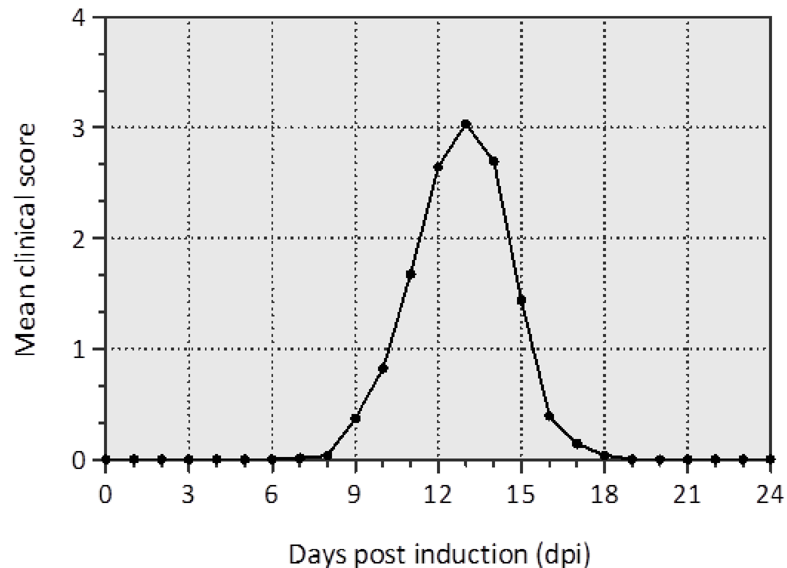

Figure 2. Quantification of neurological disability. Animals induced to a state of EAE were scored for neurological disability daily from day 0 to 24 until sacrificed. Neurological disabilities were quantified using a neurological disability assessment scale [80]. Disability values range from a clinical disability score of 0 (no disability) to 15 (maximal disability). No animals reached maximal disability. The onset of clinical symptoms appeared as early as 7 dpi (0.011 ± 0.011) and peaked at 13 dpi (3.035

±0.521) followed by complete remission by 19 dpi (score of 0). NC and AC animals did not show any neurological disability.

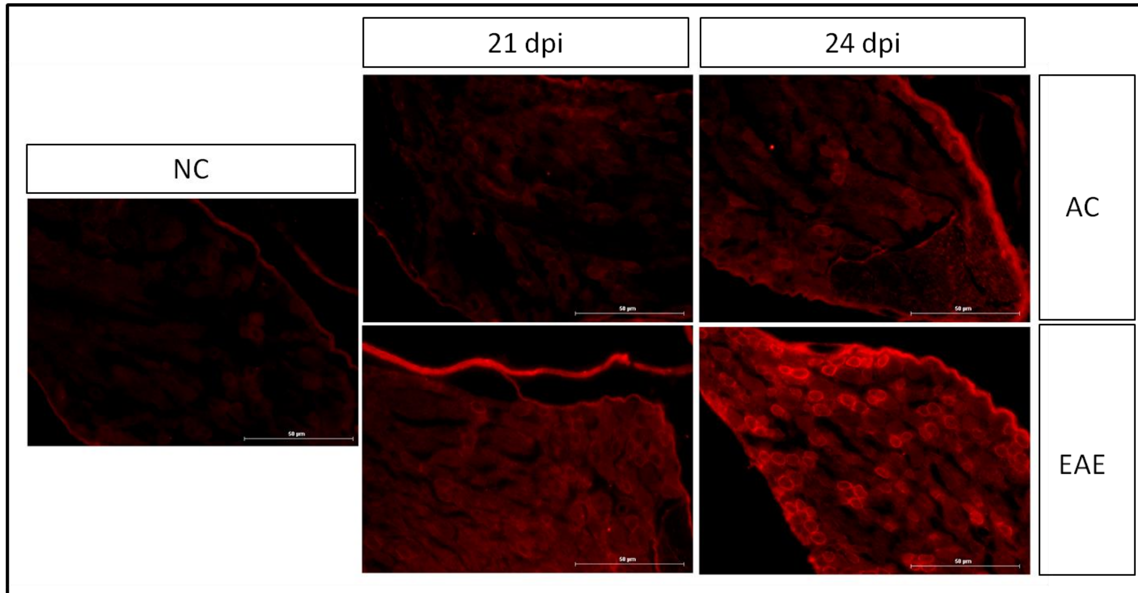

Figure 3. Qualitative analysis of NGF protein immunoreactivity changes in DRG tissue. Spatial and temporal distribution of NGF protein was assessed using IHC. 10 μm sections of DRG were stained with NGF (1:50, Santa Cruz, CN: sc-549). Total magnification of 20x using an epifluorescent microscope and camera system. Scale bars at 50 μm. Exposure time of 4s. The IHC images captured were obtained from DRG tissue subjected to identical experimental conditions and imaging procedures. Images show NGF immunofluorescent labeling (red) in NC, AC and EAE animals at 21 dpi, and AC and EAE animals at 24 dpi. There is a marked increase in NGF immunoreactivity in DRG neurons in EAE animals at 21 and 24 dpi relative to the NC and AC.

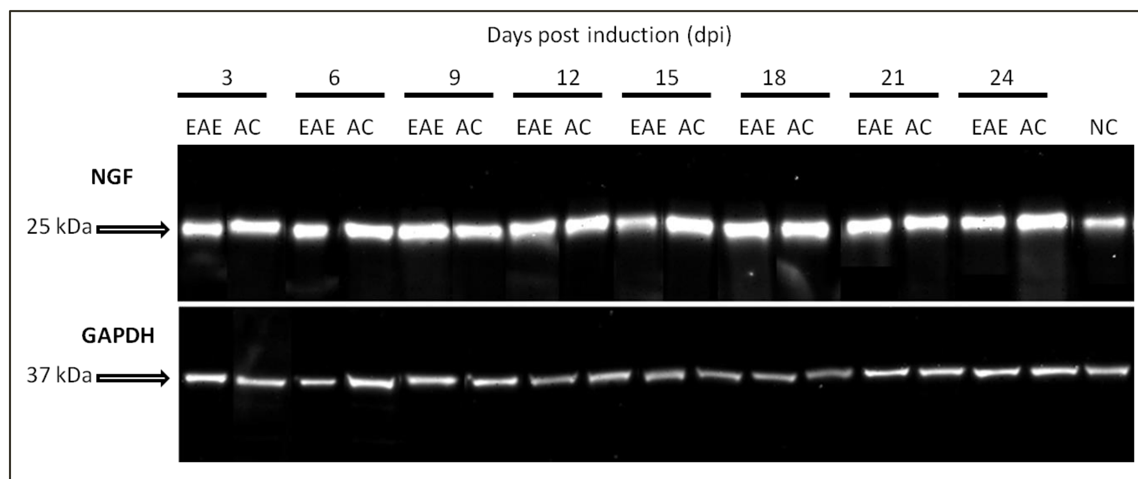

Figure 4. Representative WB of the 25 kDa pro-NGF in the DRG. An antibody against NGF (Promega, G7631, 1:500) was used to detect the 25 kDa pro-NGF in DRG tissue. This isoform was detectable in all groups and at all experimental time points. An antibody against GAPDH (Santa Cruz, sc-25778, 1:1000) was used to detect 37 kDa GAPDH to normalize NGF protein.

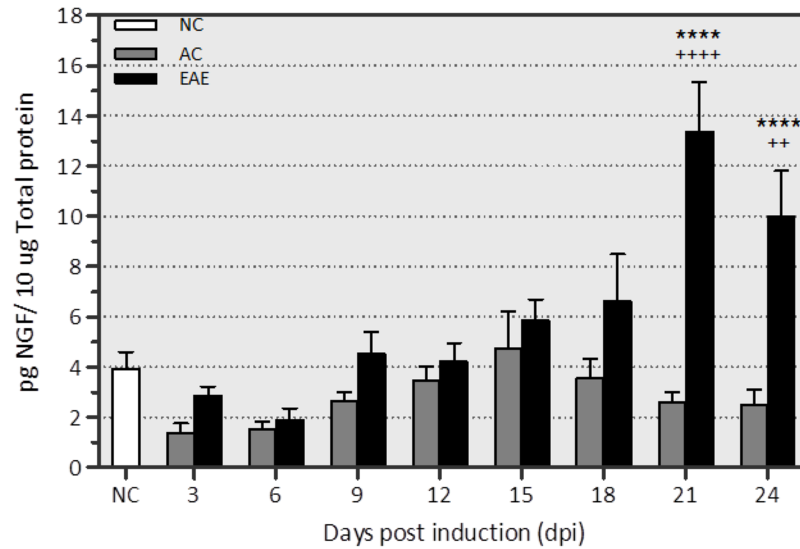

Figure 5. Quantification of the 25 kDa pro-NGF isoform in the DRG. NGF protein expression changes in lumbar DRG was quantified using ELISA in NC, AC, and EAE groups. Results are shown as pg NGF/10 μg total protein. There were delayed but significant increases in NGF expression in animals with EAE at 21 (*****P* < 0.0001) and 24 dpi (*****P* < 0.0001) compared to AC animals at 21 and 24 dpi, respectively. There were delayed and significant increases in animals with EAE at 21 (++++*P* < 0.0001) and 24 dpi (++*P* < 0.005) compared to NC. There are no significant differences between NC and AC groups. Statistics were performed using 2-way ANOVA with post-hoc Bonferroni.

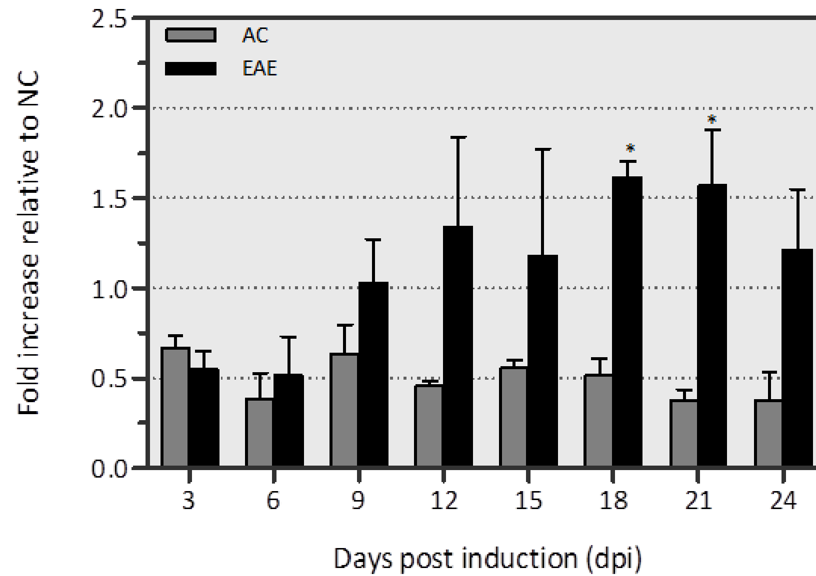

Figure 6. Quantification of NGF mRNA in the DRG. NGF mRNA expression changes in lumbar DRG were quantified using qRT-PCR in NC, AC, and EAE groups. The results are expressed as NGF mRNA fold increase relative to NC. Normalization was performed with GAPDH mRNA expression. There were significant increases in NGF mRNA expression in animals with EAE at 18 (*$P < 0.05$) and 21 dpi (*$P < 0.05$) compared to AC animals at 18 and 21 dpi, respectively. Statistics were performed using 2-way ANOVA with post-hoc Bonferroni.

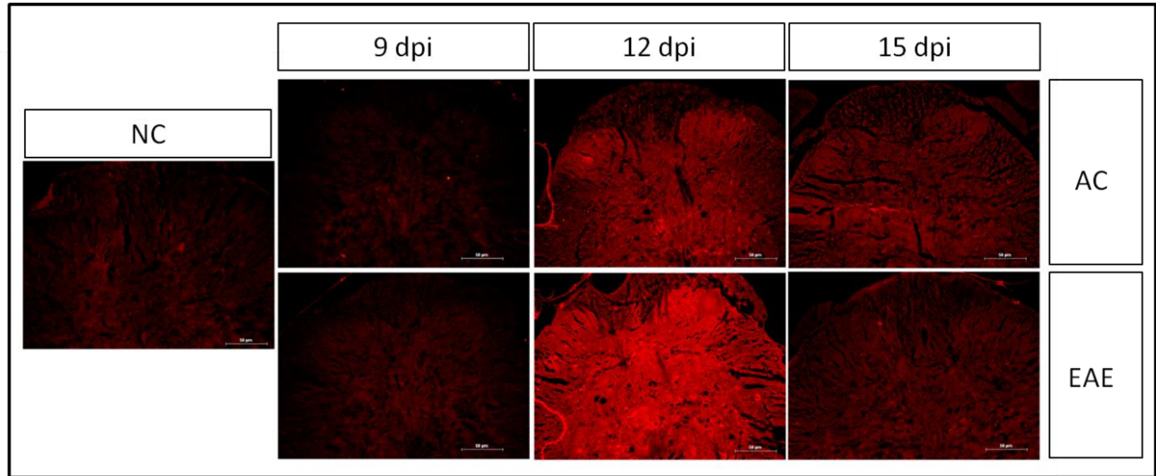

Figure 7. Qualitative analysis of NGF protein immunoreactivity changes in SC tissue. 10 μm sections of SC were stained with NGF (1:50, Santa Cruz, CN: SC-549). Total magnification of 10x using an epifluorescent microscope and camera system. Scale bars at 50 μm. Exposure time of 3s. The IHC images captured were obtained from SC tissue subjected to identical experimental conditions and imaging procedures. Images show NGF immunofluorescent labeling (red) in NC; AC and EAE animals at 9 dpi; AC and EAE animals at 12 dpi; and AC and EAE animals at 15 dpi. There is a marked increase in NGF immunoreactivity in animals with EAE at 12 dpi SC relative to the NC and AC.

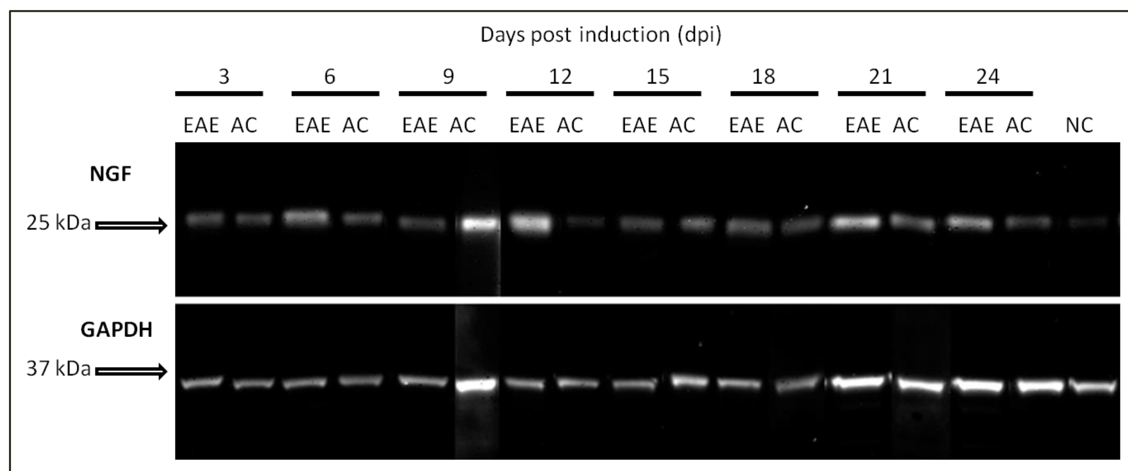

Figure 8. Representative WB of the 25 kDa pro-NGF in the SC. An antibody against NGF (Promega, G7631, 1:500) was used to detect the 25 kDa pro-NGF in SC tissue. This isoform was detectable in all groups and at all experimental time points. An antibody against GAPDH (Santa Cruz, sc-25778, 1:1000) was used to detect 37 kDa GAPDH to normalize NGF protein.

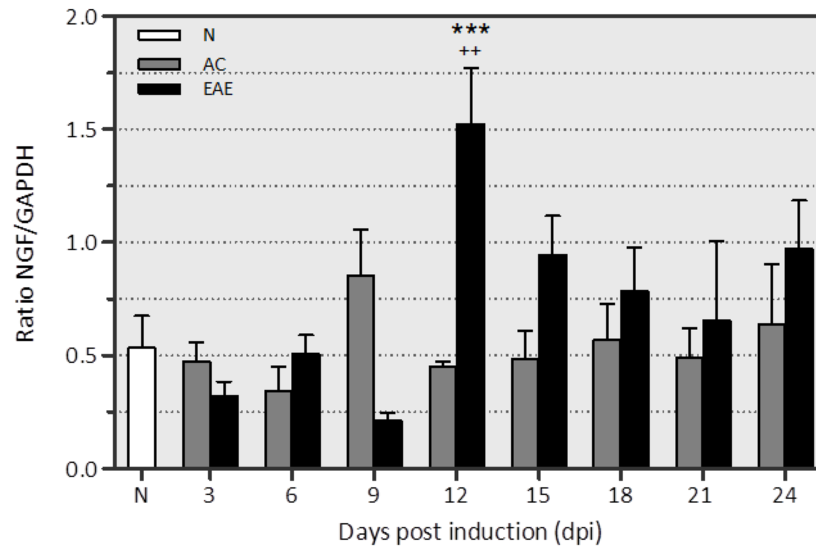

Figure 9. Quantification of 25 kDa pro-NGF protein by WB densitometry in the SC. Results are given as the ratio of NGF/GAPDH. There is a significant increase in NGF protein in animals with EAE at 12 dpi (++$P < 0.01$; *** $P < 0.001$, respectively) compared to NC and AC animals at 12 dpi, which correlated with peak neurological disability (Fig. 2). There are no significant differences between the NC and AC groups. Statistics were performed using 2-way ANOVA with post-hoc Bonferroni.

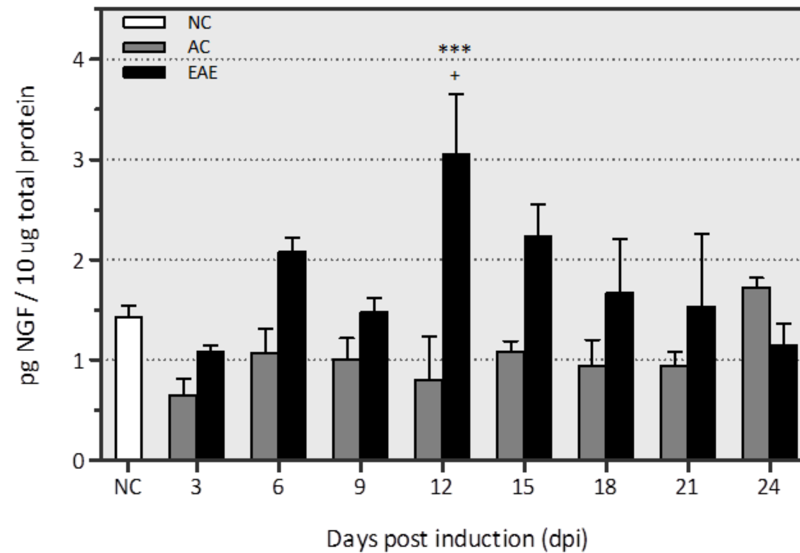

Figure 10. Quantification of the 25 kDa pro-NGF isoform in the SC. NGF protein expression changes in the SC were quantified using ELISA in NC, AC, and EAE groups. Results are shown as pg NGF/10 μg total protein. There is a significant increase in NGF protein in animals with EAE at 12 dpi (****P* < 0.001; + *P* < 0.05, respectively) compared to AC animals at 12 dpi and NC. There are no significant differences between NC and AC groups at all time points. Statistics were performed using 2-way ANOVA with post-hoc Bonferroni.

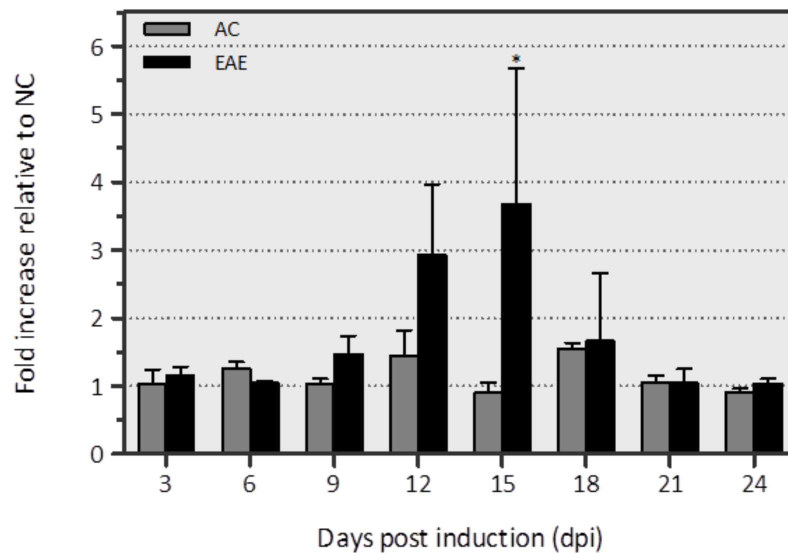

Figure 11. Quantification of NGF mRNA in the SC. NGF mRNA expression changes in the SC were quantified using qRT-PCR in NC, AC, and EAE groups. The results are expressed as NGF mRNA fold increase relative to NC. Normalization was performed with GAPDH mRNA expression. NGF mRNA expression is significantly increased in animals with EAE at 15 dpi ($P < 0.05$) compared to AC animals at 15 dpi. Statistics were performed using 2-way ANOVA with post-hoc Bonferroni.

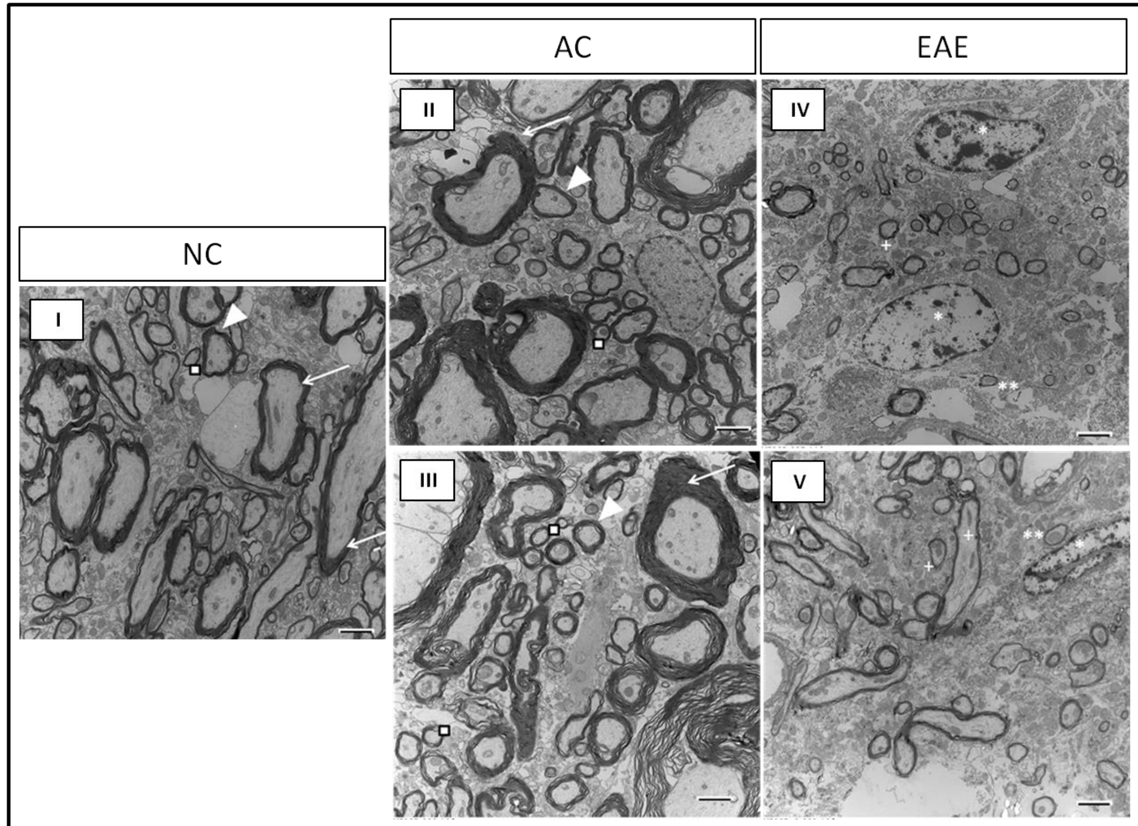

Figure 12. Changes in myelin protein structure and integrity demonstrate myelin pathology in animals induced to a state of EAE as observed by TEM. An AMT camera system was used to image SC ultrastructure under a direct magnification of 4600x. Scale bars represent 2 µm. All SC tissues were subjected to identical experimental conditions and imaging procedures. Normal myelin ensheathment of large diameter (arrow), medium diameter (arrow head), and small diameter axons (square) can be observed in SC tissue of NC **(I)** and the AC animal at 12 dpi (**II-III**). The SC tissue of the animal with EAE at 12 dpi (**IV-V)** shows a marked reduction in myelin coating of small to medium diameter axons and absence of myelin ensheathment in large diameter axons. Small diameter non-myelinated axons (**), thinly myelinated small to medium

diameter axons (+), and necrotic cells (*) can be observed in the animal with EAE at 12 dpi.

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CHAPTER 6: GLOBAL DISCUSSION AND CONCLUSIONS

6.1. OVERVIEW

MS is a chronic, progressive disease that attacks CNS myelin resulting in a wide range of symptoms that can have devastating and debilitating effects on normal daily bodily function. MS is characterized by inflammation that results in the destruction and loss of myelin, death of myelin producing OGs, and consequently, the degeneration of neuronal axons. This ultimately affects the ability of neurons to communicate with each other effectively which results in several neurological deficits that decrease the quality of life of those living with MS. Although significant advances have been made in this research field, the cure for MS still remains elusive. The identification of potential factors that can promote the repair or recovery of myelin is critical in the development of future MS therapies aimed at preventing disease progression and reversing debilitation. New ideas revolving around the role of neurotrophins, such as NGF, offer potential insights into new targeted treatment strategies for this disease. NGF may play a pivotal role in MS because of it can modulate immune processes, promote the survival of OGs, protect neurons, and contribute to re-myelination [1-8]. Despite, the plethora of evidence to support the use of NGF as a therapeutic agent for MS, its role is still not fully understood and requires further investigation.

The focus of my M.Sc. program was to explore the role of NGF during the early inflammatory stage of EAE prior to demyelination. By investigating changes in NGF

expression during the early stage of the disease process we aimed to: **1)** provide insights into disease state specific NGF mediation of inflammatory processes implicated in myelin damage and loss; and **2)** contribute to the understanding of NGF`s role in myelin repair and as a potential therapeutic agent. My research project has resulted in the publication of one review article written to provide a foundation and background for my original research, as well as, the preparation of a manuscript based on my original scientific findings surrounding the expression changes of NGF during EAE and its implications in MS. Furthermore, I have contributed to an additional two publications based on the identification of changes of fractalkine and its receptor expression during NPP and patterns of BDNF signaling via TrkB during EAE. My primary project and assistance in additional studies has allowed me to develop proficient technical research skills and contributed to my understanding and knowledge of NGF, its role in EAE, and its implications in MS and myelin repair. My research involvement in these projects has provided me the foundation to be able to problem solve and apply the principles gained to other situations. In addition, we believe the distribution of our research findings through publications and oral presentations has contributed to the advancement of NGF research in the area of MS therapeutics. Finally, our research findings provide novel information that can be built on with further research and may serve as a stepping stone for future treatment strategies aimed at improving the quality of life of MS patients.

6.2. EXPLORING THE ROLE OF NERVE GROWTH FACTOR: IMPLICATIONS IN MYELIN REPAIR

NGF was originally discovered over 60 years ago [9, 10] and in that time a large amount of work has been done to characterize this protein. In particular, its beneficial functions and involvement in CNS disorders is heavily investigated. In an attempt to consolidate all the relevant information relating to NGF in re-myelination in MS, we set out to write this manuscript as a review of NGF and its implications in myelin repair. Recent treatment strategies for MS are focusing on the use of NTs, such as NGF, in an attempt to re-myelinate lesions in the CNS known to contribute neurological deficits. Interest in NGF as a therapeutic agent is high because its beneficial effects are three-fold: **1)** it can modulate immune system processes and reduce inflammatory responses; **2)** it can promote the survival of myelinating cells of the CNS; and **3)** protect neurons and promote axonal regeneration. These functions are essential in promoting the re-myelination of lesions. Furthermore, NGF possesses immunomodulatory [11-13] and immunosuppressive functions [11, 14]. NGF can modulate immune functions by enhancing T cell-dependent antibody synthesis and is involved in acute inflammatory responses [15]. In addition, NGF can interact with microglia and immune cells [16] and interfere with their immune responsiveness [14]. Further, NGF has anti-inflammatory properties. As such, it can reduce CNS infiltration of inflammatory cells and T cell-mediated inflammation in the CNS [17, 18]. OGs and myelin are critical for the viability of myelinated axons [19-21] but are damaged or lost during the course of disease. NGF can regulate OG regeneration and the survival of OGs and OPs [6-8]. Specifically, NGF

protects OGs from TNF α -mediated apoptosis via obligatory OG survival pathways [6, 22]. NGF functions through $p75^{NTR}$ to preferentially induce TNF α signaling via TNFR2 to promote OP proliferation and myelination [23-25]. Degeneration of neuronal axons can arise during acute inflammatory and chronic demyelination [26-31] and is considered the major cause of irreversible disability [32-35]. NGF protects neurons [36-40], enhances axonal regeneration [41-43], and promotes axonal branching and elongation [44]. NGF may also indirectly promote re-myelination by inducing BDNF expression. BDNF has been shown to promote OP proliferation, OG differentiation, myelin formation, and re-myelination [45-52].

Previously, we showed that TNF α and BDNF expression are elevated in the DRG and SC during the early stages of EAE [53, 54]. Additionally, Dr Namaka's laboratory has shown the anatomical connection between the DRG and SC via the dorsal roots to be a crucial pathway for antigenically induced BDNF [54]. During development TNF α, BDNF, and NGF interact to regulate the expression of each other [24, 55]. Further, the addition of TNF α to astrocytes has been shown to increase the expression of BDNF [56]. In addition, BDNF can induce the secretion of NGF, which subsequently promotes preferential TNF α signaling via TNFR2, making TNF α protective [57, 58]. Moreover, TNF α induces NGF expression [59] and NGF can also induce the production of BDNF [60, 61]. This led us to propose a critical interconnected signaling triad between TNF α, BDNF, and NGF that may regulate inflammatory responses that may affect myelin repair and re-myelination processes in the SC.

Currently, available MS treatment options are limited to use in the relapsing remitting form of MS (RRMS). At present, there are no treatments for secondary progressive MS (SPMS). Once patients enter SPMS the disabilities that result from myelin damage remain permanent, therefore, treatment strategies must target the early phases of the disease. Although the exact mechanism of action of IFN-β products are unknown, they are thought to induce NGF expression [62]. However, while several studies support the use of NGF as a remyelinating therapeutic agent for MS, the development of NGF therapies have been limited due to its poor pharmacokinetic profile and challenges in administering NGF without unwanted side effects [63-66]. Different methods of administration have been investigated with promising results including gene delivery, viral vectors, encapsulated cell bio-delivery, and mimetic peptides [63, 67-73]. While significant advances have been made in the area of MS therapeutics, and in particular, MS treatments relating to NGF, future research is still required to fully understand the role of NGF in MS to better determine courses of treatment. Further, the intricate relationship between cytokines and neurotrophins has yet to be studied as a plausible mechanism of myelin repair. Specifically, more studies relating to NGF need to be conducted during early inflammatory EAE since immune-mediated and inflammatory processes start during the earliest stages of MS [74] and axonal degeneration can take place during acute inflammatory demyelination [26, 27]. By identifying early changes in NGF expression we can provide information regarding the role of NGF in neurological recovery during inflammatory processes contributing to myelin damage.

6.3. NERVE GROWTH FACTOR GENE AND PROTEIN EXPRESSION INCREASES DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: IMPLICATIONS IN MULTIPLE SCLEROSIS AND MYELIN REPAIR

Our published review of NGF and its implications in MS and myelin repair allowed us to set a conceptual foundation for our study on NGF expression changes during early, inflammatory EAE. One of the largest motivations behind this study was the limited availability of treatment options for MS. Although, current MS therapies are immune system modifying, there is still a great need to develop dual approach therapeutics aimed at reducing inflammation and promoting re-myelination. As previously discussed in **Chapter 2** and summarized in **section 6.2**. there is considerable evidence to support the use of NGF as a therapeutic measure for MS because of its ability to regulate the immune system, protect OGs and neurons, and promote re-myelination.

As already referenced, our previous publications demonstrated that TNF α and BDNF is up-regulated during EAE in the DRG and SC. In this current study we sought out to investigate NGF signaling in the DRG and SC during EAE. Inflammatory processes contribute to myelin damage and NGF expression changes during acute inflammation in EAE could provide a better understanding of the role of NGF in MS and its treatment.

In this study we employed the Lewis rat MBP model of EAE to induce inflammation. Animals induced to a state of EAE were monitored for neurological disability. Disability was quantified as a measure of successful antigenic induction which reflected the EAE disease process and was subsequently correlated to changes in NGF protein and gene expression. All animals with EAE showed similar disease progression, normally beginning with tail weakness and progressing to complete paralysis of the hind limbs. We determined the peak of disease occurred at 13 days after induction. In animals induced to a state of EAE we showed significant increases in NGF gene and protein expression in the DRG that correlated with complete neurological recovery. We believe this may be due to the ability of NGF to promote the recovery phase of EAE by down-regulating pathogenic TNF α signaling via TNFR1. In the presence of NGF, TNF α preferentially signals through TNFR2, making TNF α protective rather than pathogenic [23-25, 57, 58]. As such this would minimize the maintenance of TNF α-mediated myelin damage, and consequently, reduce TNF-induced BDNF expression. In addition, we determined a significant increase in NGF gene and protein expression in the SC of animals induced to a state of EAE, however, mRNA expression is delayed relative to protein. We believe this acute increase in NGF protein in the SC may be released from adjacent supportive as a response to counter the detrimental effects of TNF α. Furthermore, we believe DRG-derived BDNF may be the critical factor that actually regulates the local release of NGF from neurons and surrounding supportive cells in the SC. This study further identified the 25 kDa pro-NGF isoform as a biologically active form during EAE. In addition, we uncovered several NGF isoforms of larger molecular weight that could possibly be explained by post-translational modifications. We were not

able to detect the mature isoform of NGF using three separate antibodies (1:200, Santa Cruz, CN: sc-549; 1:200, Santa Cruz, CN: sc-548, 1:500, Promega, CN: G1132). Other studies have reported difficulty in detecting the mature isoform (13 kDa). Our findings suggest that this isoform may not be the predominant form during the EAE disease process. In fact, one particular group determined the proNGF (32 kDa) to be the predominant isoform in the brain [75]. Finally, this study recognizes the effects of acute inflammation on the structure and integrity of myelin during EAE. In animals with EAE at peak neurological disability we observed a mark reduction and absence of myelin ensheathment in various sized axons compared to the healthy and non-disease controls. We consider this change in myelin integrity to be the result of inflammatory processes and contribute to the neurological disability seen in animals with EAE. Further, we correlated this change in myelin structure with the increases in NGF expression. An increase in NGF expression during time points associated with changes in myelin ensheathment indicates NGF may be involved in ameliorating these processes, thereby contributing to neurological recovery, as indicated by improvements in neurological disability.

This study provides insights into the role of NGF during acute inflammatory EAE that may be exploited when developing novel MS treatments. As previously mentioned, this study utilized the MBP Lewis rat EAE model. The rat model of EAE is the preferred animal model to study the inflammatory processes involved during disease progression, as well as, to study changes in target proteins during EAE inflammation. This particular feature of this model is pertinent in studying early changes in NGF, particularly, if we are

interested in early changes that contribute to the amelioration of disease symptoms. However, this model has limited use if we are interested in studying the direct effects of NGF on the processes of demyelination and re-myelination. Since the Lewis rat MBP model employed in this study is a monophasic acute inflammatory model of EAE, it would be beneficial then to extend this research into a biphasic, chronic demyelinating model of EAE such as the MOG C57/Bl6 EAE model, which would more closely resemble the relapsing remitting MS disease course. In fact, we have now extended our research efforts to include a fully functional MOG-induced EAE mouse model. Using a mouse model also opens up new research possibilities that include the use of knockouts that are not as readily available in rat species.

6.4. SUMMARY

MS is characterized by inflammation, demyelination, and subsequent axonal demyelination. There is no cure for MS and current treatment strategies are limited to modulating inflammatory processes, slowing disease progression, and decreasing disease severity. The most beneficial treatment for MS would be one that is anti-inflammatory and re-myelinating. Re-myelination is of utmost important in improving the quality of life of MS patients because it would not only prevent disease progression, but it could potentially reverse permanent disability. As previously mentioned in length, NGF is a very attractive candidate for novel treatment strategies for MS because it is not only an immunomodulator, but it is also capable of promoting re-myelination in the CNS. The research accomplished during my M.Sc. program has been heavily focused on characterizing patterns of NGF expression during acute inflammation during EAE that may contribute to neurological recovery and may be implicated in myelin repair. We provide new information regarding NGF expression changes in the DRG and SC that supports our proposed critical signaling triad. In addition, this study contributes to the advancement of NGF research relating to MS treatment. Lastly, we provide information that can serve as a basis for additional research that may lead to the development of novel re-myelinating treatment strategies. As mentioned, one interesting study would be to use a chronic demyelinating animal model of EAE to study the direct effects of NGF on re-myelination. I have also contributed to two other manuscripts focused on fractalkine and fractalkine receptor signaling during NPP and BDNF signaling via TrkB during EAE. All these studies have helped me to develop scientific skills and have contributed to my

understanding and appreciation for MS and the difficulties we face as scientists in finding a cure.

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